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CELLULAR MECHANISMS OF DECREASED MYOCARDIAL RESPONSIVENESS  
TO CATECHOLAMINES DURING ENDOTOXICOSIS IN THE RAT

by

Fred D. Romano

A Dissertation Submitted to the Faculty of the Graduate School  
of Loyola University of Chicago in Partial Fulfillment  
of the Requirements for the Degree of  
Doctor of Philosophy

May

1985

DEDICATION

TO CORI AND DANI

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I would especially like to thank my advisor, Dr. Stephen B. Jones. His patience, unlimited encouragement, advice, and friendship were essential to the realization of my goal.

My appreciation goes to the members of my committee for their expert advice and guidance and for a few laughs along the way.

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## VITA

The author, Fred Daniel Romano, is the son of Vincent John Romano and Carmela (Arena) Romano. He was born March 3, 1951, in Brooklyn, New York.

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The author married Corinne (Warren) Romano on April 5, 1982 and they have one daughter, Danielle Marie.

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## CHAPTER I

### INTRODUCTION

Bacteremia is an ongoing phenomenon that is usually handled by a healthy person without any difficulty or external manifestation of the problem. Occasionally the bacterial insult becomes overwhelming and induces a sequence of changes, the so-called septic state. Depending on the ability of host to respond appropriately to the insult, the changes may be mild and reversible or severe and irreversible - culminating in death in septic shock. In the clinical setting, sepsis often progresses significantly before it is recognized. In many cases even aggressive treatment with antibiotics, cardiopulmonary support, or steroids fail to curtail progression into a shock state and death ensues (92,97). In this regard, approximately 1% of hospital patient admissions annually results in gram-negative sepsis; thirty to fifty percent of these cases will be fatal. McCabe et al (91) projected that at these rates as many as 300,000 episodes of sepsis and more than 100,000 fatalities from gram-negative infections occur in the U.S. each year. Therefore gram-negative sepsis and the consequences of sepsis are significant health problems.

Studies performed to characterize the basic pathophysiologic processes of sepsis have been conducted for nearly 100 years and yet

mechanisms responsible have not been satisfactorily elucidated. Some of the lack of insight centers around the experimental model of sepsis. At this time there is no universally accepted model of sepsis, although some recent models may duplicate the symptoms of clinical sepsis better than earlier models (136). One particularly reproducible model of sepsis is the endotoxin injection model. Endotoxin is a lipoprotein-carbohydrate complex found in the cell wall of gram-negative bacteria. The bacterial lipopolysaccharide consists of a highly variable outer region composed of repeating oligosaccharide units and a relatively constant core region containing a small number of amino sugars and the biologically active lipid A moiety. Endotoxin can be extracted from the cell wall by a number of methods; most commonly including treatment with trichloroacetic acid (Boivin method) or phenol-water (Westphal method).

Endotoxin has been found to cause a shock syndrome and eventually death in a variety of experimental animals. It has also been implicated as the precipitating factor in experimental animal sepsis (139). Approximately thirty years ago it was suggested that human gram-negative shock was also caused by endotoxins. Spink et al (120) postulated that the antibacterial action of antibiotics destroys viable organisms, liberating antigenic material, presumably endotoxin. This theory was supported by clinical studies of Borden and Hall (12) and Braude et al (14) who observed severe shock in patients infected with gram-negative organisms. The relationship of endotoxin to sepsis has not been unanimously accepted, and many investigators maintain that

the cardiovascular and metabolic responses to endotoxin in experimental animals do not necessarily correlate with the clinical picture of sepsis. These discrepancies may be related to species variability or time course of the response. Recently, however, evidence has accumulated that strongly suggests the pathophysiologic role of endotoxin in human gram-negative bacteremia. Ziegler et al (142) have significantly improved survival of patients exhibiting gram-negative bacteremia, by treating them with human antiserum produced against the core of endotoxin. Similarly, freeze-dried human plasma rich in anti-LPS immunoglobulin G was used to treat septic shock in obstetric and gynecological patients (71). Treated patients had a significantly improved survival rate and the survivors had a shorter hospital stay than survivors of conventional methods. In close agreement with these studies, Pollack et al (112) have observed enhanced survival in Pseudomonas aeruginosa septicemia in patients who exhibited increased plasma concentrations of endogenous core-specific antibodies. The data from these studies supports the use of the endotoxin model as relevant to the study of the pathophysiology of sepsis.

The condition of endotoxemia is associated with a plethora of metabolic, humoral and hemodynamic alterations (58). Of particular consequence is the depression in myocardial performance that develops during endotoxemia. Historically, a decrease in venous return, due to peripheral pooling, has been considered to contribute to the failure of the pumping action of the heart, especially in the early stages of endotoxemia. More recently, evidence has emerged that suggests that

myocardial dysfunction also occurs as a result of an intrinsic defect. The mechanisms of this dysfunction are currently under investigation in numerous laboratories.

Activation of the sympathetic nervous system is an important compensatory mechanism that proceeds during endotoxemia. Early in the septic insult sympathetic activity supports the metabolic and cardiovascular adjustments to endotoxemia. Eventually the sustained sympathetic hyperactivation becomes detrimental to the animal and contributes to the pathogenesis of the endotoxemia (78). There is some evidence that high levels of catecholamines directly cause lesions in the heart (102) and catecholamines are also implicated as causing myocardial adrenergic receptor dysfunction in similar stress states (131,138). In addition, it is believed that the myocardium becomes refractory to catecholamine stimulation during endotoxemia (1,6,107,114). Apparently the beta-adrenergic effector system in the heart is modified in endotoxemia, the precise mechanisms of such changes have not been elucidated.

Recent technological advances have contributed substantially to the understanding of adrenergic receptor structure and function in normal and pathophysiological situations (45). With the apparent alteration in beta-adrenergic receptor mediated mechanisms developing during endotoxemia, these techniques are directly applicable to the analysis of myocardial beta-adrenergic receptor characteristics in this pathophysiological condition.

Therefore, this dissertation: 1) reviews the evidence for myocardial dysfunction in endotoxin shock and the related mechanisms for the dysfunction, 2) reviews the role of the sympathetic nervous system in supporting the cardiovascular system in shock and how this support may be modified, 3) develops an approach to studying adrenergic post-synaptic mechanisms in the heart during endotoxemia, 4) presents experimental findings pertinent to the questions raised, and 5) relates the present results to the existing literature.

## CHAPTER II

### REVIEW OF RELATED LITERATURE

#### A. Evidence for Myocardial Dysfunction in Endotoxiosis

The role of the heart in the pathogenesis of endotoxin shock is still somewhat controversial, however it is generally accepted that there is a decline in myocardial performance as endotoxiosis proceeds (76). Myocardial performance can be defined as the ability of the heart to pump blood to the periphery as determined by preload, afterload, heart rate, and contractility. Therefore myocardial performance is influenced by extracardiac as well as intracardiac factors. There is still considerable debate whether the decrease in myocardial performance during endotoxiosis is a result of intrinsic myocardial dysfunction or if it is due to alterations in the peripheral vasculature. In all probability both myocardial failure and vascular collapse contribute to the decline, with the time course of the endotoxiosis dictating which is the predominant factor.

Initially, it was believed that the decline in myocardial performance during endotoxiosis was related solely to a decrease in venous return (34). Hinshaw et al (50) noted vascular changes, particularly increased venous resistance, during irreversible endotoxic



shock. They suggested that the vascular changes could account for the hypotension and pooling that occurs during endotoxin shock. It is likely that these changes contribute to a decreased venous return leading to a decline in myocardial performance

Experiments by Lillehei et al (77) supported the role of the vasculature in myocardial performance alterations. They observed decreased cardiac output and visceral organ blood flow in dogs during the latter stages of endotoxemia even while blood pressure was supported at normal levels by increased peripheral resistance. This was attributed to the destructive effect of catecholamines on the microcirculation. Treatment with plasma or low molecular weight dextran concomitantly with phenoxybenzamine, an alpha-adrenergic receptor antagonist, brought peripheral resistance and plasma catecholamine levels back towards normal and increased venous return, cardiac output and organ blood flow. They believed that any decrease in myocardial function during endotoxemia was a result of peripheral vascular defects.

Early evidence for myocardial dysfunction in endotoxemia was reported by Solis and Downing (118). Left ventricular function was studied in an autoperfused feline heart preparation. They observed that within 15-30 min of endotoxin administration, both stroke volume and ejection rate for a given left ventricular end diastolic pressure at constant heart rate and aortic pressure were reduced, as compared to

control. The reduction in ventricular contractility was attenuated by the infusion of inotropic agents as late as one hour after administration of endotoxin. They suggested that the decrease in ventricular contractility may contribute to the circulatory failure in endotoxin shock but did not offer any mechanism of the deterioration.

Goodyer, in 1967 (40), using anesthetized dogs, correlated ventricular function changes with measurements of systemic oxygen debt and metabolic indices of myocardial anoxia. Myocardial failure was not indicated until late in endotoxin shock and only at a time when the dogs were in severe oxygen debt. When dogs received beta-adrenergic receptor blockade along with endotoxin administration ventricular function was impaired. Thus the myocardium may be undergoing a slow but progressive deterioration that is masked by increased sympathetic drive. Goodyer concluded that myocardial damage manifests when sympathetic drive fails or is no longer effective. The cause of the myocardial impairment could not be related to anoxia or acidosis in this study and therefore remained in question.

In contrast to these findings, Hinshaw et al (51) found no deleterious effect of propranolol on canine isolated heart preparations after endotoxin administration. Cardiac performance was unimpaired after endotoxin even in the presence of beta-adrenergic blockade. The dose of endotoxin used in this study was approximately one-fourth of that used by Goodyer (40), a discrepancy that serves to demonstrate the difficulty in comparing data between studies. In a subsequent study with the same

preparation as above, in which they controlled aortic pressure and cardiac output, Hinshaw et al (52) found no direct toxic effect of endotoxin on the canine myocardium within 3.5 hours. However they suggested that prolonged systemic hypotension may precipitate cardiac failure due to decreased coronary perfusion pressure and flow. It is important to note that at this time the Hinshaw group used a relatively short experimental period to assess cardiovascular parameters during endotoxemia.

Berk et al (5) also administered propranolol to endotoxic dogs and observed an increased survival rate in the propranolol-treated dogs as compared to those that received only endotoxin. They concluded, similarly to Lillehei et al (77), that the beneficial effects of adrenergic antagonists were at the level of the microvasculature and not at the heart, in that arteriovenous shunts in the lung and splanchnic area remained closed and pooling of blood was avoided. Myocardial function was not directly assessed in by Berk et al (5).

Cann et al (16) studied the cardiovascular response of nonseptic and septic dogs to various perturbations. Using volume loading of dogs with dextran to increase preload, no clearcut evidence was found for any change in cardiovascular performance in sepsis. However, when the dogs received an infusion of isoproterenol just prior to the dextran, the cardiac index of the septic dog did not increase as seen in the control state. In fact the cardiac index-mean right atrial pressure curve of the septic dog with the beta-adrenergic agonist was not significantly

different from septic dogs with only volume expansion. When control and septic dogs underwent beta-adrenergic blockade before dextran expansion the control dogs were able to raise their cardiac index to levels comparable to dextran treatment alone despite no significant change in heart rate. In contrast the septic dogs, which also had no heart rate increases could not increase cardiac index even at high filling pressures. These data suggest that myocardial function, especially those mechanisms relating to increasing stroke volume, are depressed during sepsis. These data also suggest that the septic heart is dependent on adrenergically mediated increases in heart rate to increase cardiac output.

In contrast to their earlier studies Hinshaw et al (53) noted irreversible myocardial failure in isolated dog hearts six to nine hours after endotoxin injection. They observed a markedly elevated left ventricular end diastolic pressure, decreased maximum change in left ventricular pressure and the need of inotropic agents to support the endotoxic heart. At this time they could only conjecture as to the precipitating factor in the myocardial dysfunction.

The interpretation of data regarding in vivo myocardial function during endotoxemia poses a number of problems, one of these related to the sensitivity of the methods used. Many methods may be influenced by alterations in cardiac loading or peripheral vascular function (37). Recently, Guntheroth et al (41) have observed an early sustained reduction in myocardial contractility in dogs, receiving twice the

LD<sub>100</sub> of endotoxin, as assessed by state-of-the-art techniques (e.g. segment length-pressure loops). They suggest that the depression in contractility may not be apparent in most preparations in that the myocardium is relatively unloaded due to diminished preload and afterload. In good agreement with this study, Goldfarb et al (38) also noted an early diminished myocardial contractility in dogs that did not survive an LD<sub>50</sub> dose of endotoxin. They also employed more suitable methods for assessing contractility. Presumably the techniques used in these studies are not influenced by extra-cardiac modulation of contractility and indicate a definite decrease in myocardial function. However, others (66) using similar techniques could not find any decrease in myocardial function in the dog until the agonal stages of endotoxiosis. Therefore, the time course of the decline in myocardial function remains in doubt.

Assessment of myocardial function in vivo is also influenced by the endotoxic milieu, which may contain stimulants and/or depressants that mask any change in intrinsic function. Therefore, in vitro studies may be important to determining myocardial function in shock. Parker and Adams (106) observed myocardial contractile dysfunction in guinea pig atria, taken from animals that had received endotoxin 16 hours previously, noting a decreased isometric contractile tension and maximum rate of tension development in endotoxic tissue as compared to control. In contrast, McCaig et al (93) could find no significant contractile, electrophysiological, or ultrastructural differences between cardiac muscle removed from cats five hours after endotoxin administration and

from control cats, not administered endotoxin. Both studies could find no direct effect of endotoxin, in vitro, on myocardial function. The difference in species, dose of endotoxin, and time course of the endotoxiosis make it difficult to assess the relative degree of shock in each group, although the severity of endotoxiosis appears to be greater in the cats, which showed no myocardial depression.

### B. Factors Influencing Myocardial Performance During Endotoxiosis

While there is still some controversy over the existence of myocardial dysfunction during endotoxiosis, the majority of evidence supports the view that heart function deteriorates by the intermediate and late stages of endotoxiosis. The proposed mechanisms of the dysfunction are numerous and it is apparent that myocardial failure is a result of some combination of alterations.

#### 1. Coronary blood flow and related problems

Compromised coronary blood flow resulting in a decreased myocardial oxygen supply has been demonstrated during endotoxiosis. Hinshaw et al (56) suggest that this is due to a decrease in coronary perfusion pressure associated with the sustained systemic hypotension that develops rapidly after endotoxin administration. Others (10,15) have observed a significant increase in coronary vascular resistance and a decrease in coronary flow in endotoxin shock. Increases in coronary resistance are thought to be mediated by angiotensin II, which presumably is released during the hypotensive phase (73).

Efforts to preserve myocardial function by augmenting coronary blood flow with vasodilators have been equivocal. Bohrs et al (10) were not able to increase myocardial function after increasing coronary blood flow with dipyridamole, however Peyton et al (110) found that myocardial function was improved with nitroprusside administration. The difference in response to these substances may be related to their effects on preload and afterload. Augmentation of coronary flow by mechanical means has been somewhat more successful. Studies by Elkins et al (29) and Bruni et al (15) have both demonstrated protection of the myocardium when coronary flow was artificially maintained.

Myocardial dysfunction has been related to myocardial fluid disturbances and intra-cardiac ionic imbalances. A study by Mela et al (95) demonstrated inter- and intrafiber edema in isolated dog ventricles 4-7 hours after endotoxin administration. These ventricles were considered to be in failure as determined by elevated end diastolic pressures. Similar findings were also reported by Coalson et al (20). Hinshaw (57) suggests that this edema may exacerbate an increase in coronary capillary and myocardial cell membrane permeabilities.

In addition to, and possibly due to the myocardial edema, alterations in ionic concentrations have been indicated in the endotoxic heart. Experiments by Hinshaw et al (54) suggest that there is an alteration in myocardial calcium handling during endotoxemia. Treatment of dogs with digoxin, a cardiac glycoside which is thought to increase intracellular calcium levels, attenuates the myocardial dysfunction of endotoxemia. This may be due to increasing the

availability of calcium to the contractile apparatus.

The appearance of abnormally elevated extracellular potassium concentrations secondary to decreases in coronary flow, during endotoxemia may also contribute to myocardial depression (2). High extracellular potassium concentrations are known to reduce myocardial contractility by accelerating repolarization and shortening the plateau of the action potential, which together reduce calcium entry into the cell (64).

## 2. Humoral factors

Myocardial function is also thought to be modulated by circulating humoral agents. The myocardial depressant factor (MDF) reported by Lefer is probably the best known substance of this category. According to Lefer (72) MDF is formed by the ischemic splanchnic viscera and activated by lysosomal hydrolases primarily from the liver. MDF has been demonstrated to directly decrease tension development in isolated papillary muscles (72). In addition, Carli et al (17) have demonstrated that the serum from septic human patients attenuates the chronotropic response to isoproterenol in rat myocytes. There is some evidence that MDF depresses fast channel activation in the ventricular myocardium (25). In contrast, Hinshaw et al (51) have not been able to detect a myocardial depressant factor in endotoxic dogs. This suggests that MDF is not produced under all situations or that the concentration of MDF that is effective in vitro is not attained in vivo. Therefore the significance of MDF production during endotoxemia is still conjecture.



Endogenous opiates may also depress myocardial function. Reynolds et al (113) have reversed endotoxin-induced decreases in myocardial function by administration of naloxone, an opiate receptor antagonist. While most opiate receptors have been identified in the central nervous system, there is evidence that high affinity opiate receptors exist on myocardial membranes (117). Whether the myocardium is the site of endogenous opiate action is yet to be determined.

Plasma catecholamines (4,63,99,121) and sympathetic discharge (105) to the heart are increased during endotoxemia. There is evidence to suggest that high catecholamine concentrations may cause lesions in the heart and contribute to myocardial dysfunction (102). The importance of this mechanism in endotoxemia has not been determined.

Histamine release is an early event in endotoxin shock, the degree being dependent upon the dose of endotoxin (133). Blood histamine levels have been demonstrated to be inversely correlated with survival in rats (116). Treatment of endotoxic animals with antihistamines protects myocardial function (68). However, the depression of myocardial function by histamine does not appear to be a direct effect but rather is mediated by the decrease in coronary blood produced by histamine (109).

Two other mediators produced during endotoxemia are also gaining attention in regards to their possible cardiovascular effects. Leukotrienes, which are metabolites of arachidonic acid and related to

prostaglandins, are thought to increase in plasma during anaphylaxis and septic shock (42). Since leukotrienes are reported to depress myocardial contractility in vitro (75), they may contribute to the myocardial depression evident in endotoxemia, however this is merely speculative. The macrophage mediator, interleukin-1, is also released during infection and appears to have multiple biologic activities (28). Whether this substance interacts with the myocardium to any significant extent is yet to be determined.

The direct effects of endotoxin on myocardial function have also been studied. In vivo studies have usually been unable to show a direct effect of endotoxin (52). The in vitro effects of endotoxin on the heart have been nonexistent for the most part (93,106) although a few studies have indicated that endotoxin influences some myocardial metabolic variables and subcellular organelles (69,83).

### 3. Subcellular derangements

Reductions in myocardial function, regardless of the external stimuli, are ultimately related to activation and function of the contractile apparatus. McCaig and Parrat (94) demonstrated that endotoxic cat hearts are not as sensitive to increasing extracellular calcium concentrations as control hearts. Likewise, Krause and Hess (69) have demonstrated a decrease in canine sarcoplasmic reticulum (SR)  $\text{Ca}^{+2}$ -ATPase activity and calcium uptake rate after in vivo administration of endotoxin. This would especially affect the rate of relaxation of myocardial muscle. In addition to SR changes, myofibrillar

ATPase activity was also significantly decreased in the same model. Failure of the sarcoplasmic reticulum to accumulate calcium would increase cytosolic concentrations and thereby stimulate mitochondrial calcium sequestration (48). The increase in mitochondrial calcium concentration would then depress ATP production and decrease intracellular ATP concentrations. Mela et al (95) found no changes in mitochondrial energy production in endotoxic dog hearts although there were some ultrastructural abnormalities. The data from Krause and Hess (69) suggests that elevated intracellular concentrations of calcium are deleterious to myocardial function. In contrast to these data, studies by Hinshaw et al (54) and Somani and Saini (119) indicated that increasing intracellular levels of calcium will preserve myocardial function. The differences between Krause and Hess and these other groups are not readily apparent at this time, although it may be a matter of which calcium handling process in the myofibril is most affected during the endotoxic insult.

Sarcolemmal membrane alterations have also been demonstrated. Cardiac electrophysiological parameters are somewhat modified during exposure to septic serum as evidenced by depressed action potential amplitude, duration, resting membrane potential, upstroke velocity in dog heart membranes (25). Rat hearts show similar depressions except that the action potential duration is lengthened (17). Relevant to this data, Ghosh and Liu (33) report that ouabain-sensitive sodium pump activity is impaired in dog hearts during endotoxin shock as reflected by diminished rubidium uptake by heart slices. They have also indicated

that the fluidity of the myocardial membrane is modified in the shock state (84), which may affect ionic movements across the sarcolemma and adenine nucleotide transport in mitochondria.

### C. Sympathetic Nervous System Support of Cardiovascular Function

Beyond the myriad of extra- and intra-cardiac mechanisms contributing to the myocardial dysfunction of sepsis is the hypothesis that throughout the phase of progressive depression of myocardial contractility, the adrenergic system supports myocardial performance. The importance of sympathetic nervous system support is proposed by a number of investigators. Solis and Downing (118) reported that infusions of isoproterenol were necessary to increase left-ventricular performance, in the one hour endotoxic cat, to control levels. Similarly, Hinshaw et al (53) found that they could temporarily restore function with beta-adrenergic agonists to failing dog hearts in the intermediate stages of endotoxicosis. Dogs that are given beta-adrenergic receptor blockers early in sepsis (16) or endotoxicosis (40) are not able to maintain myocardial function at control levels or able to increase function to meet the demands of the shock state. Cats that receive alprenolol prior to endotoxin administration had no recovery of myocardial contractility and died within 4 to 6 minutes (107). In addition, adrenalectomized dogs or rats are more sensitive to lethal doses of endotoxin (70,121).

Concomitant with the apparent requirement for adrenergic support

of the myocardium is a generalized increase in sympathoadrenal discharge during endotoxemia (99,105,121). Thus the myocardium is stimulated by catecholamines originating from the adrenal medulla or sympathetic nerve endings. This activity is elevated throughout the septic or endotoxic insult. In theory there are always adequate concentrations of catecholamines to support the myocardium. There is evidence, however, that sympathetic nerve endings in the heart and the adrenal medulla become depleted (104, unpublished observations) at the terminal stages of endotoxemia but that there is still significant release of catecholamines at that time as determined by plasma levels (63).

#### D. Myocardial Response to Catecholamines During Endotoxemia

The response of the myocardium to beta-adrenergic agonists is essential to maintenance of myocardial performance and possibly to the survival of the endotoxic animal. Paradoxically, the inotropic response of the myocardium appears to be depressed in the later stages of sepsis or endotoxemia. Parrat (107) reported that the inotropic and chronotropic response to an infusion of norepinephrine or epinephrine to cats was markedly reduced 2-3 hours after endotoxin administration. Parrat and Winslow (108) also observed attenuated myocardial responses to quozadine, a phosphodiesterase inhibitor, in endotoxin shock in the cat. Somani and Saini (119) demonstrated that the cardiovascular effects of a dopamine infusion were diminished by two hours after endotoxin administration in the dog. Archer and associates (1) also reported attenuated responses in the isolated working dog heart preparation. Four

to six hours after endotoxin administration epinephrine did not restore  $-dP/dt$  to normal values. However, they failed to show any significant change in  $-dP/dt$  in response to epinephrine in the control hearts. Data from in vivo and isolated heart studies is tempered somewhat by the possibility that high levels of endogenous catecholamines may mask the effects of exogenous catecholamines. However Sugerman et al (129) found that increased serum catecholamine levels of conscious dogs in septic shock did not correlate significantly with cardiac index, which implied a decreased responsiveness to catecholamines. Additionally, Baghat et al (6) observed that left atrial strips from endotoxin-treated guinea pigs, suspended in Tyrode's solution, had a reduced sensitivity to norepinephrine and tyramine, while responding normally to stretch. This data suggests that the phenomenon of decreased responsiveness to catecholamines is not due to a factor present in the septic plasma as proposed by Carli et al (17) nor is it an artifact due to increased plasma catecholamine levels.

The inotropic effects of beta-adrenergic agonists are mediated by intracellular cAMP (101), therefore the decreased responsiveness is likely due to some alteration in the beta-adrenergic receptor-adenylate cyclase system and/or the processes controlled by this system. There is evidence that the beta-receptor-adenylate cyclase system of the dog liver is altered during endotoxemia (32,85), however studies of this nature have not been extended to the myocardium.

McCaig and Parrat (94) suggest that the decreased response to

catecholamines is not due to any alteration in the adenylate cyclase activating system, but reflects an alteration in calcium-mediated mechanisms. Evidence has also been discussed that demonstrates contractile apparatus depression. However, the depression in each system is not mutually exclusive and together serve to amplify myocardial dysfunction in shock. Loss of responsiveness to catecholamines is not directly responsible for decreased myocardial contractility in endotoxycosis. Catecholamine-stimulated mechanisms merely serve to delay or diminish the dysfunction originated by other lesions.

Considering the importance of the adrenergic nervous system in supporting the myocardium during sepsis or endotoxycosis, experiments to define adrenergic input into the heart and to assess the status of the beta-adrenergic receptor-adenylate cyclase system are clearly necessary for understanding the mechanism of myocardial dysfunction under these conditions.

## CHAPTER III

### BETA-ADRENERGIC STIMULATION OF MYOCARDIAL CYCLIC AMP IN ENDOTOXIC RATS



## INTRODUCTION

Endotoxycosis with ensuing cardiovascular shock is characterized by a variety of hemodynamic responses (58). The initial response to an endotoxin challenge is a rapidly developing hypotension, decreased venous return, and depressed cardiac output. A compensatory period usually follows the hypotensive phase where blood pressure returns towards normal. Sympathoadrenal homeostatic mechanisms are active during this period however adrenergic discharge may not be stimulated solely by the systemic hypotension and associated reflex mechanisms (121). Depending upon the severity of the insult the animal may recover completely or progress to circulatory collapse.

Jacobson (61) described an "adrenergic theory of shock" whereby the sympathetic nervous system is activated as a compensatory mechanism in shock to aid in the restoration of blood pressure. Such compensation however, results in the sympathetic nervous system becoming overstressed which eventually contributes to the pathogenesis of the shock state. While this theory is attractive, supporting experimental data are not unequivocal. Bhagat et al (7) reported that chemical sympathectomy with 6-hydroxydopamine did not alter the severity of endotoxin shock in monkeys and Trippodo and Traber (132) had similar findings in the dog. In contrast, sympathectomy with 6-hydroxydopamine in the rabbit protected against the lethality of endotoxin (11). Selective surgical denervation of the abdominal viscera or spleen in dogs also protected

from the injury or lethality of endotoxic shock (103,141). Thus, the pathogenic effects of sympathetic nervous system hyperactivation are not global and may be limited to specific organs or tissues. Furthermore, there may be a distinction between the degree of pathogenicity of the alpha-adrenergic limb of the sympathetic nervous system as compared to beta-adrenergic limb.

Cyclic AMP (cAMP) is the mediator of the beta-adrenergic limb of the sympathetic nervous system and regulates many intracellular processes related to metabolism and myocardial function (101). The effects of endotoxin on cAMP accumulation and adenylate cyclase activity have been studied in a number of metabolically active tissues (8,35,122). Most recently, Ghosh and Liu (32) have described endotoxin attenuation of catecholamine-stimulated adenylate cyclase activity in dog liver membranes. Alterations in the hepatic response to catecholamines during sepsis has profound effects on the metabolic status of the organism and may ultimately determine survival. Likewise, adrenergic support of the myocardium is critical during shock. There is evidence that the heart has a decreased response to exogenous catecholamines during the latter stages of endotoxemia (1,6,107), which contributes to declining myocardial performance. This may be related to an inability to generate cAMP in this tissue, similar to that seen in the liver. In this regard, the status of myocardial cyclic nucleotide levels during endotoxemia have not previously been reported.

The purpose of the present study was to determine the time course of sympathetic nervous system activation and neurotransmitter exhaustion in various tissues of the rat during endotoxemia and to assess the activation of the cAMP generating system in the heart under the same conditions.

## METHODS

### Animal model

Male Holtzman rats (300-400 g) were used in all experiments. Rats were maintained on Wayne Lab Blox and tap water ad libitum for at least 7 days after they were received from the supplier (Holtzman Co., Madison, WI). Food was available to the animals at all times prior to the experiments.

Endotoxin (S. enteritidis, lipopolysaccharide B) was purchased from Difco Laboratories, Detroit, MI. The same batch of endotoxin (#703252) was used for all experiments. Endotoxin was prepared daily in 0.9% saline. Lightly etherized rats were injected with endotoxin (16.7 mg/kg) via the dorsal vein of the penis. This dose elicited greater than 80% lethality within 24 hours. Control rats were injected intravenously with 0.9% saline.

### Ventricular Slice Preparation

Rats were decapitated at the appropriate times and the hearts rapidly removed and placed in ice-cold Krebs-Ringer bicarbonate buffer (KRB) which contained (mM): NaCl, 118.5; KCl, 4.8;  $\text{KH}_2\text{PO}_4$ , 1.2;  $\text{MgSO}_4$ , 1.2;  $\text{CaCl}_2$ , 3.6; glucose, 5.5; and  $\text{NaHCO}_3$ , 23.8. The KRB was gassed with 95%  $\text{O}_2$ -5%  $\text{CO}_2$  and the pH adjusted to 7.4. The ventricles were sliced in a cold room with a Stadie-Riggs microtome (Arthur H. Thomas Co., Philadelphia, PA) to a thickness of 0.5 mm. Slices were incubated in

oxygenated KRB at 37°C, pH 7.4, for 15 minutes in a metabolic shaker while being continuously gassed with 95% O<sub>2</sub>-5%CO<sub>2</sub>. At the end of the incubation period, 100 ul of 2.5 x 10<sup>-5</sup>M isoproterenol or vehicle (0.1% ascorbic acid) was added to the incubation mixture. After 45 sec. the slices were removed from the incubate, blotted dry and frozen between brass clamps previously cooled in liquid nitrogen. Tissues were stored in liquid nitrogen until processed for cAMP determination.

### Cyclic AMP Assay

Frozen samples were weighed and placed in 10 volumes of ice-cold 10% trichloroacetic acid (TCA). Tissues were homogenized at 4°C for 20 sec. with a Brinkman Polytron at setting 6. The homogenates were centrifuged at 1900 x g for 20 min. One ml of supernatant was extracted four times with 4 volumes of water-saturated ethyl ether. The extracted aqueous phase was frozen and lyophilized. The residue was dissolved in 1 ml of 50 mM sodium acetate buffer, pH 6.2, and used directly in the assay.

Cyclic AMP was measured by the method of Steiner et al (126). Assay kits were purchased from New England Nuclear, Boston, MA.

### Tissue Catecholamine Content

Rats were killed by decapitation and the hearts, spleens, and adrenals rapidly removed and frozen by compression between brass clamps previously cooled in liquid nitrogen. Tissues were stored in liquid nitrogen until assayed for catecholamines.

Norepinephrine and epinephrine content were measured by the method of Crout (21). Frozen tissue was weighed and homogenized in 10 ml of ice-cold 10% TCA. Homogenates were centrifuged at 1800 x g for 20 min. Catecholamines were isolated from the protein-free supernatant by column chromatography with acid-washed alumina. Recovery of catecholamines from the column was routinely 80% efficient; sample values are reported as uncorrected for percent recovery. The column eluate was analyzed for catecholamines by forming the trihydroxyindole derivative of the bioamine and measuring its fluorescent intensity. The procedure was automated as described by Jellinek et al (62).

#### Glucose Measurement

Plasma glucose concentration was determined by the glucose oxidase method using a Model 23A glucose analyzer (Yellow Springs Instrument Co., Yellow Springs, OH).

#### Data Analysis

Statistical comparison of two groups was performed by an independent Student's t test. Comparison of more than two groups was by independent analysis of variance. Significant F tests were followed by the mean separation technique of least-significant difference. A P value  $<0.05$  was considered to be the minimum for statistical significance.

## RESULTS

Plasma glucose concentrations, tissue catecholamine contents and myocardial cAMP contents are shown in Table I. Plasma glucose levels are used as an indicator of the severity of the shock state (30). At one hour after endotoxin administration, given levels were more than double the control value, however the glucose values declined rapidly and by 4 hours were significantly less than the control value.

Splenic norepinephrine levels were significantly decreased in the endotoxic rat by two hours and remained at the same level for the duration of the experimental period.

Adrenal norepinephrine content decreased during the endotoxic insult, however it was significantly different only at 4 hours. In contrast, adrenal epinephrine content was reduced by 1 hour and continued to decline throughout 6 hours of endotoxemia.

Myocardial norepinephrine remained constant throughout the experimental period. Myocardial cAMP levels were significantly reduced early in endotoxemia but were not different from control at the later stages.

A problem in performing such a time course study is that not all animals respond to the endotoxic insult with the same rate. Thus, the

TABLE 1.  
TIME COURSE OF ENDOTOXEMIA (5 mg/300 g)

	TREATMENT	1 Hr.	2 Hrs.	4 Hrs.	6 Hrs.
Plasma Glucose (mg/dl)	Saline	145.7 ± 6.4(7) *	129.4 ± 3.1(7) *	139.4 ± 3.2 (7) *	133.3 ± 3.9(7) *
	Etox	183.4 ± 22.3(8)	204.7 ± 31.4(10)	71.4 ± 10.8(9)	80.1 ± 10. (1)
Spleen NE (μg)	Saline	732 ± 0.1(7)	.918 ± .17(6) *	.64 ± .05(7) *	.847 ± .14 ( )
	Etox	709 ± .6(8)	.564 ± .04(10)	.53 ± .03(8)	.566 ± .05 (1)
Adrenal NE (μg/pr)	Saline	.41 ± .06(7)	.44 ± .04(7)	.62 ± .07(6) *	.60 ± .18(7)
	Etox	.37 ± .02(7)	.45 ± .07(10)	.43 ± .05(8)	.37 ± .05(10)
Adrenal EPI (μg/pr)	Saline	1.81 ± 0.19(7) *	2.26 ± .2(7) *	2.15 ± .19(6) *	2.1 ± .19(7) *
	Etox	1.39 ± 0.11(7)	1.25 ± .12(10)	.63 ± .06(8)	.40 ± .04(10)
Myocardial NE (μg/g)	Saline	.820 ± .05(7)	.825 ± .07(6)	.752 ± .03(6)	.805 ± .06(7)
	Etox	.794 ± .06(8)	.700 ± .04(9)	.795 ± .13(6)	.892 ± .07(11)
Myocardial cAMP (pmol/g)	Saline	1955 ± 92(7) *	1748 ± 32(6) *	1655 ± 126(6)	1739 ± 50(7)
	Etox	1488 ± 69(8)	1410 ± 47(9)	1563 ± 42(8)	1585 ± 52(11)



values determined during the time course reflect the average of animals in different states of endotoxiosis and only the survivors at each time point are used. Therefore, the changes in the measured variables in severely shocked animals may be masked by values from rats that are more resistant to the insult. To circumvent this problem, tissues were collected from a group of animals when they were in the terminal stage of endotoxiosis, regardless of the duration of the endotoxiosis. The terminal stage of endotoxiosis was assessed as that time when the animal loses the righting reflex. These data are depicted in Table II. Plasma glucose concentrations, spleen norepinephrine, adrenal norepinephrine and epinephrine, and myocardial norepinephrine contents were all significantly diminished in the endotoxic tissues. In addition, the cAMP content of the ventricles from endotoxic rats was reduced 19% from control.

Figure 1 shows the isoproterenol-stimulated cAMP content in rat ventricular slices 0.5 hours after the IV administration of saline or endotoxin. There were no significant differences in either the basal levels or stimulated levels of cAMP between control and endotoxic animals. At 3 hours after endotoxin administration there was no difference in basal levels, however, the isoproterenol-stimulated cAMP content in the endotoxic tissue was not greater than basal (Fig 2). Cyclic AMP content increases by 23% in 3 hour control tissue stimulated by isoproterenol. At the terminal stage of endotoxiosis (approximately 5 hours after endotoxin administration) tissues from saline-treated animals responded to isoproterenol stimulation with a 29% increase in

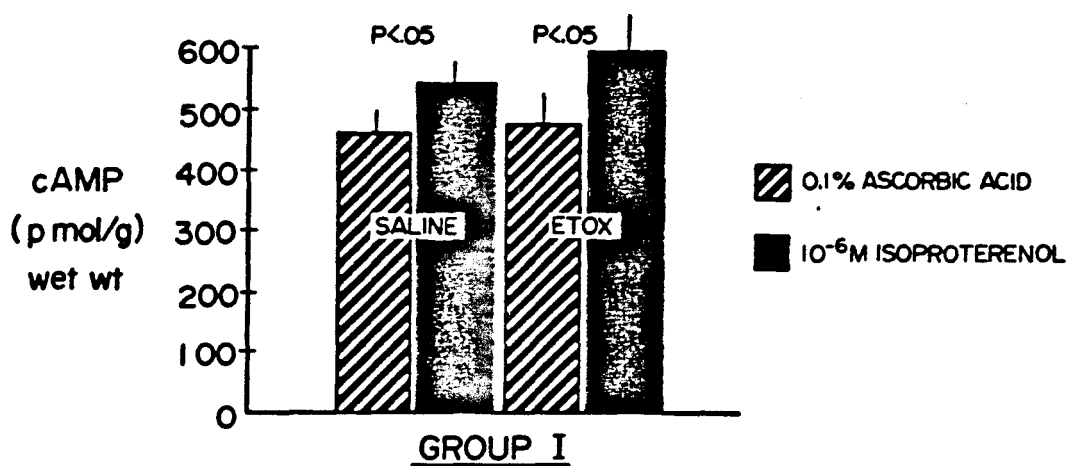
TABLE II  
PHYSIOLOGICAL VARIABLES OF FED RATS FOLLOWING IV  
ADMINISTRATION OF SALINE OR ENDOTOXIN

	<u>Saline (N=8)</u>	<u>ETOX (N=14)</u>
TIME TO DEATH (mins.)	330 $\pm$ 40	311 $\pm$ 30
Plasma Glucose (mg/dl)	154 $\pm$ 3	23 $\pm$ 3*
SPLEEN NE (ug/g)	.77 $\pm$ .08	.36 $\pm$ .04*
ADRENAL NE (ug/pr)	.49 $\pm$ .04	.33 $\pm$ .03*
ADRENAL EPI (ug/pr)	2.2 $\pm$ .1	.41 $\pm$ .07*
MYOCARDIAL NE (ug/g)	.97 $\pm$ .05	.38 $\pm$ .06*
MYOCARDIAL cAMP (pmol/g)	2044 $\pm$ 47	1658 $\pm$ 43*

\*p<0.05

Figure 1.

Cyclic AMP (pmol/g wet weight) content of ventricle slices, before and after isoproterenol stimulation ( $10^{-6}$  M), from control or treated rats 0.5 hours after saline or endotoxin administration. Each bar represents the mean  $\pm$  SEM for 5-7 preparations, each from a different rat.



HEARTS REMOVED 0.5 HOURS  
POST I.V. ADMINISTRATION OF  
SALINE OR ENDOTOXIN

Figure 2.

Cyclic AMP (pmol/g wet weight) content of ventricle slices, before and after isoproterenol ( $10^{-6}$  M) stimulation, from control or treated rats 3 hours after saline or endotoxin administration. Each bar represents the mean  $\pm$  SEM for 5-6 preparations, each from a different rat.

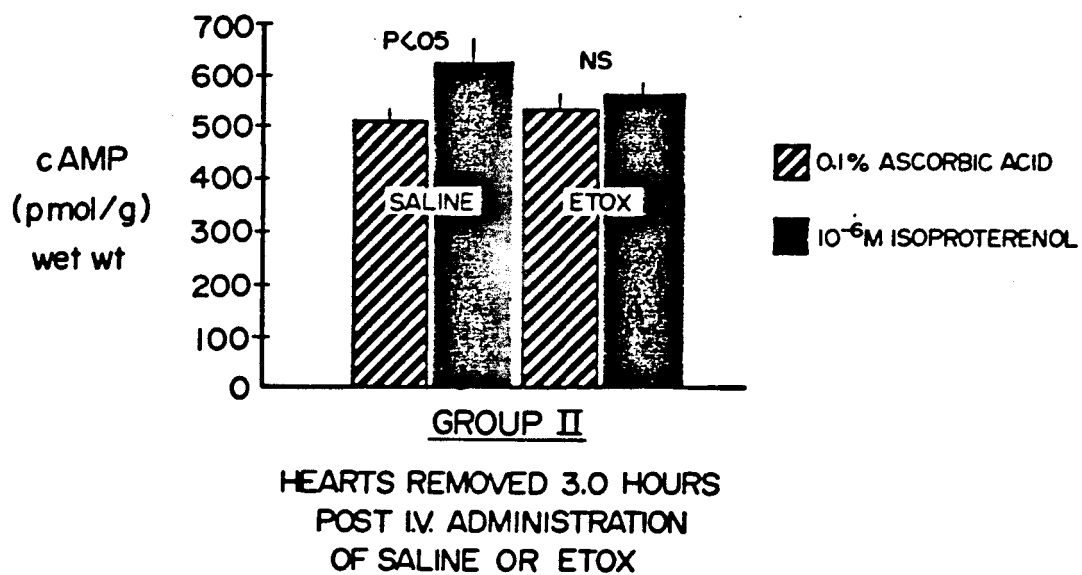
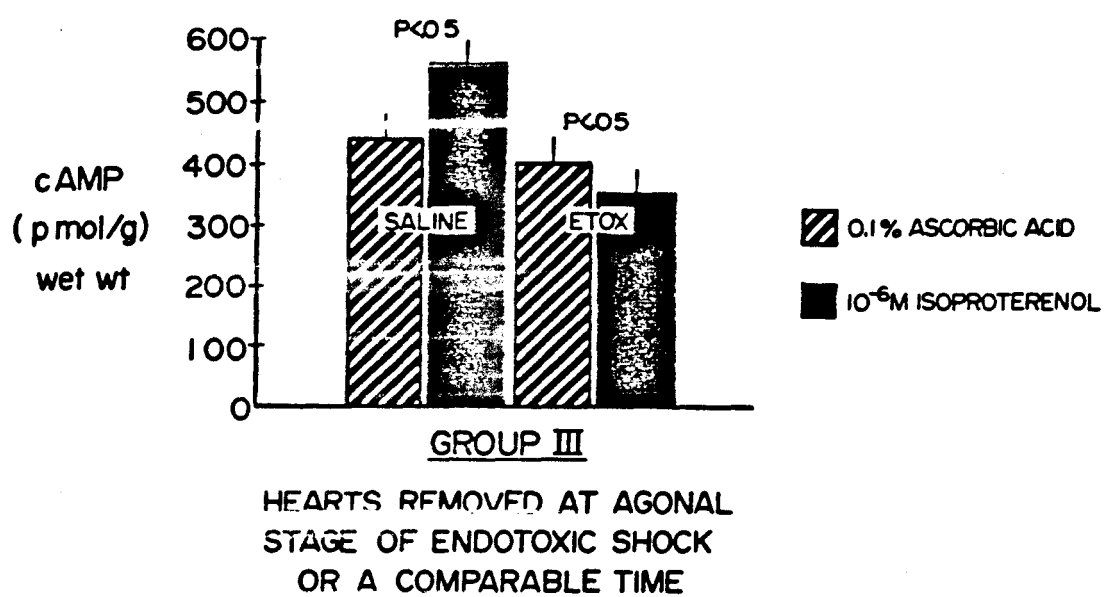


Figure 3.

Cyclic AMP (pmol/ g wet weight) of ventricle slices, before and after isoproterenol ( $10^{-6}$  M) stimulation, from control or endotoxin-treated rats at the terminal stage of endotoxemia. Each bar represents the mean  $\pm$  SEM for 8 preparations, each from a different animal.





CAMP content, whereas tissue from endotoxic animals failed to respond positively.

## DISCUSSION

Intense activation of the sympathetic nervous system is generally accepted as a manifestation of sepsis and endotoxemia (63,99,105). Sympathetic activity appears to have beneficial circulatory and metabolic effects early in endotoxemia, but as the endotoxemia progresses, continued adrenergic stimulation may become detrimental to the survival of the animal (78). Mechanisms which mediate beneficial vs. detrimental actions of sympathetic activity have been suggested in a number of studies. Increased survival in endotoxemia followed treatment with alpha-adrenergic receptor blockers or surgical denervation (77,103), however, beta-adrenergic receptor blockade with propranolol significantly decreased time to death in endotoxic rats (31). Adrenergic blockade affects both metabolic and cardiovascular processes. The predominant action of alpha-adrenergic blockade on the cardiovascular system, is to antagonize the vascular effects of norepinephrine and epinephrine, whereas beta-blockade interrupts adrenergic input to the myocardium. It appears that the alpha-receptor mediated mechanisms are injurious while beta-receptor mediated mechanisms ameliorate the effects of endotoxin. However, both alpha- and beta-adrenergic components may have beneficial as well as detrimental effects depending on the stage of endotoxemia or the particular tissue being stimulated.

The data presented in Table I are suggestive of nonuniform

activation of nerves to major organs during the endotoxic insult. Adrenal stores of epinephrine are depleted early in the endotoxemia, implying intense stimulation of the medulla and a rise in epinephrine output. Presumably this results in the markedly elevated plasma concentration of epinephrine observed in endotoxic rats (63). Nykiel and Glaviano (99) described a similar rise in epinephrine output in endotoxic dogs and found that the release was due to a neurogenic mechanism that appeared to be activated by a decrease in arterial blood pressure. A similar mechanism may be in effect in the rat in that a significant hypotension has been observed in the rat within 10 minutes after an  $LD_{50}$  dose of endotoxin (63). It is believed that the heart undergoes a progressive deterioration during endotoxemia, however sympathetic stimulation via beta-adrenergic receptors appears to augment contractility and maintain myocardial performance adequate for the needs of the peripheral tissues. Release of excessive amounts of epinephrine from the adrenal medulla may be the primary mechanism for the support of myocardial contractility during the endotoxic insult. In agreement with this concept, Spink et al (121) reported that the lethal course of canine endotoxin shock was accelerated in adrenalectomized animals.

Splenic norepinephrine becomes depleted within two hours after the onset of endotoxemia (Table II). Norepinephrine turnover in the spleen during low dose endotoxin has been reported to increase three fold (105). This suggests that depletion of norepinephrine is mediated by nerve-stimulated release. Pardini et al (104) have also shown reuptake to be attenuated in the spleen at the terminal stages of

endotoxic shock. If such changes occur earlier in the endotoxemia, this could account for the norepinephrine depletion in the spleen observed by 2 hours in the present study. Immediate activation of sympathetic fibers to the spleen may be part of the neurogenic response to the hypotension that develops soon after endotoxin administration. Presuming that the spleen is representative of innervation to the peripheral vasculature in other areas, activation of adrenergic fibers to this tissue would mediate vaso- and venoconstriction and augment return of blood pressure towards normal. However, sustained constriction appears to be harmful in that venous return is compromised, due to pooling of blood, and certain vascular beds becoming ischemic (50,77).

Although myocardial norepinephrine is not depleted within six hours after endotoxin administration it must be noted that these animals are all survivors and have plasma glucose levels well above what may be indicative of a shock state (30). Myocardial norepinephrine depletion may just be a terminal event as suggested by the data in Table II. Sympathetic nerve activity to the heart is presumably increased during endotoxemia (105), and this may be more important for chronotropic and dromotropic mechanisms that control cardiac output rather than for potentiation of inotropic properties (3), whereby circulating epinephrine will have more significant inotropic stimulating properties.

There is some evidence that sympathetic fibers to the heart are differentially controlled from those of the vasomotor system or adrenal medulla (137) and recently Hintze and Vatner (59) have described

differential peripheral sympathetic activation during hemorrhage. The cardiovascular adaptations to endotoxemia are very similar to those of hemorrhage and the sequence of neurotransmitter depletion of the tissues in the present study indicate that sympathetic nerve discharge to the periphery may have different intensities and times of activation.

The values of tissue cAMP content of the heart during endotoxemia also raise some interesting questions. Early in endotoxemia the tissue content is lower than control but there is no difference between the survivors at 6 hours and saline controls (Table I). At the terminal stages the cAMP content of endotoxic ventricle is significantly (19%) lower than control (Table II). The cAMP content in the in situ ventricle reflects generation as well as degradation of cAMP. Therefore, even though there may be an increased production of cAMP in the heart during the first few hours of endotoxemia this may not be reflected by tissue levels. This could be explained by the interaction of a variety of hormones and mediators, that are released or produced as a result of endotoxemia, with the cAMP-generating system. Yelich and Filkins (140) have reported rats to be hyperinsulinemic during the first two hours after endotoxin administration. Insulin is known to have anti-epinephrine action in adipose tissue and liver by increasing phosphodiesterase (PDE) activity in these tissues (39). Thus the hyperinsulemia may increase cAMP degradation in the heart although production is increased. At the terminal stage of endotoxemia, plasma insulin is near normal (140) and presumably would not have a significant effect on PDE activity. Therefore, the decrease in cAMP content at that

time is most likely due to a decrease in production.

We examined the integrity of the beta-adrenergic receptor-adenylate cyclase system in the myocardium by using a ventricular slice preparation. Three hours after endotoxin administration ventricular slices from the endotoxic rats did not show increased cAMP in response to isoproterenol-stimulation. Basal levels were not different between the groups, suggesting that there was no increase in degradation in the endotoxic tissue. The loss of responsiveness in the endotoxic tissue could be due to a decrease in substrate availability, inhibition of the generating system by an endogenous mediator, a lesion in the generating system, or a combination of the three. ATP levels in a variety of tissues have been shown to decrease in shock or low flow states (19). While the status of myocardial energy charge in endotoxemia has not been reported, myocardial hypoperfusion has been noted for some time (10,15,29). Depression of ATP levels may occur in the heart during the late stages of endotoxemia, concomitant with the hypoperfusion, thereby decreasing substrate available to adenylate cyclase.

A number of cardiodepressant factors have been reported to be produced during sepsis or endotoxemia (36). Generally these substances are thought to have a direct effect on the myocardium, however Carli et al (17) have described a product in human septic serum that also inhibits the chronotropic stimulatory effects of isoproterenol on rat myocardial cells. More recently, the macrophage mediator, interleukin-1,

has been implicated as contributing to the pathogenesis of infection (28). Whether this substance has any inhibitory interaction with the beta-adrenergic receptor-adenylate cyclase system is yet to be reported.

A number of studies (1,6,107) have suggested that there is a decreased functional responsiveness of the septic or endotoxic myocardium to catecholamines. This phenomenon may be related to the failure of the endotoxic ventricle to accumulate cAMP. The generation of cAMP is a result of catecholamine-beta-adrenergic receptor interactions on the myocardial cell membrane and is dependent upon 1) the number of available receptors to bind the agonist, 2) the affinity of the receptor for agonist, 3) the efficiency of coupling between the receptor and adenylate cyclase, and 4) the functional status of the cyclase. Once produced, cAMP mediates the phosphorylation of intracellular proteins important to the myocardial contractile process. If this system is modified by endotoxin or a mediator of endotoxiosis the cAMP production may be decreased and the ability of adrenergic stimulation to support cardiac contractility compromised. Thus the preservation of cardiovascular performance by stimulation of cardiac beta-adrenergic receptors in the early stages of endotoxiosis may be a beneficial homeostatic mechanism. However the efficiency of this mechanism may deteriorate during the development of endotoxic shock.

The catecholamines that support the myocardium early in endotoxiosis may also be responsible for the decreased functional responsiveness to catecholamines observed at the terminal stage. The

concept of tachyphylaxis or desensitization is firmly established as a catecholamine-induced mechanism in cardiovascular tissue (127). The sustained elevated levels of sympathetic nervous system activity to the heart in the form of norepinephrine release or circulating epinephrine could possibly reach levels high enough to initiate this change. Therefore it is conceivable that the beneficial effects of catecholamines on the heart become detrimental when beta-adrenergic receptor stimulation is excessive or sustained.

The specific role of the sympathetic nervous system in the pathogenesis of sepsis or endotoxemia is not universally accepted and the data from the present study leaves many questions unanswered. Clearly more studies are needed to determine the stimulus for the sympathetic hyperactivation and the relative outflow to the peripheral organs and tissues. Additionally the mechanisms by which the seemingly beneficial actions of catecholamines become deleterious must be elucidated.



## CHAPTER IV

# ALTERATIONS IN BETA-ADRENERGIC STIMULATION OF MYOCARDIAL ADENYLATE CYCLASE IN ENDOTOXIC RATS

## INTRODUCTION

Gram-negative sepsis and the related consequences have been documented to be persistent health problems (91) deserving of aggressive scientific investigation. Furthermore, recent evidence (71,112,142) has linked bacterial endotoxins to the clinical expression of sepsis, which supports the use of the endotoxic animal model for studying the basic pathophysiologic process of septic shock.

Myocardial dysfunction is generally accepted as a manifestation of sepsis and endotoxemia (58,76), but the time course and mechanisms of failure are not readily agreed upon. Some investigators have observed myocardial depression as soon as one hour after endotoxin administration (38,41) whereas others have not seen changes for five or six hours after endotoxin (53) and still others find myocardial failure is only a terminal event (66). It has been suggested (40) that the myocardium of endotoxic animals may undergo a slow but progressive deterioration that is masked by increased sympathetic drive. Indeed sympathetic nervous system activity is markedly increased during endotoxemia (105) or sepsis (4) and it has been shown that myocardial function is depressed further during endotoxemia or sepsis when beta-adrenergic receptor antagonists are administered (16,40). Therefore it appears that beta-adrenergic stimulation is important in supporting cardiac function during sepsis.

Reduced responsiveness to catecholamines in the latter stages of endotoxycosis has been observed by a number of investigators. Parrat (107) reported that the effects of epinephrine and norepinephrine infusions on blood pressure, heart rate, cardiac output, myocardial blood flow and left ventricular  $dP/dt_{\max}$  were markedly reduced 2-3 hours after endotoxin administration to anesthetized cats. Recovery of the response to norepinephrine was associated with a general circulatory improvement. Bhagat et al (6) observed a reduced inotropic response to norepinephrine in left atrial strips from endotoxin-treated guinea pigs. Archer and associates found that four to six hours after endotoxin treatment norepinephrine infusions did not restore positive or negative  $dP/dt$  values to normal in the isolated dog heart. Recently, Sugerman and co-workers (129) have noted that increased serum catecholamine levels of septic dogs did not correlate significantly with cardiac index or systemic vascular resistance. The reduction in response to catecholamines in all of these studies appears to unmask myocardial deterioration that may manifest in circulatory collapse.

The mechanisms of such changes in responsiveness are unknown. A number of investigators have proposed the existence of a myocardial depressant factor (MDF) in the serum of septic or endotoxic subjects (17,74). This substance appears to attenuate the myocardial response to catecholamines in vitro (17). Other investigators (55) have not been able to observe the proposed actions of an MDF-type substance in their preparations and therefore the MDF theory does not have unanimous support. Since it is known that adrenergic stimulation of myocardial

contractility is mediated by the beta-adrenergic receptor-adenylate cyclase system and that this system has been observed to be modulated under a number of physiological or pathophysiological situations (45), the adrenergic mechanism itself may be the point of alteration. A modification of any of the components of the system could result in a decreased response to catecholamines. Preliminary studies (114) have shown a reduced tissue content of cAMP in response to isoproterenol stimulation in rat ventricular slices 5 hours after in vivo endotoxin administration. This may be the result of a decrease in cAMP generation or increased degradation of cAMP. In either case augmentation of myocardial contractility would be compromised.

The purpose of the present study was to examine myocardial adenylate cyclase activation via beta-adrenergic receptors during various stages of endotoxemia in the rat and to determine if changes in responsiveness develop in this system. Our data suggest that the integrity of beta-adrenergic receptor-adenylate cyclase system is compromised in the late stages of endotoxemia and that this may correlate with myocardial failure which is characteristic of endotoxin shock.

## METHODS

Animal Model

Healthy male Holtzman rats (Holtzman Company, Madison, WI) weighing between 300 and 400 g were used in this study. Rats were housed in wire-mesh cages at 22° C ambient temperature with a 12-hr light dark cycle and provided with food (Wayne Lab Blox) and water ad lib. Rats were kept in this manner for at least one week prior to use in experimental protocols.

Endotoxin (Difco Laboratories, Detroit, MI) was purchased as a single batch (703252) of Boivin lipopolysaccharide from Salmonella enteritidis. Endotoxin for injection was prepared fresh daily in 0.9% saline (5 mg/ml). Rats were lightly anesthetized with ether and injected with endotoxin (16.7 mg/kg) intravenously via the dorsal vein of the penis. Previous experiments had shown this dose of endotoxin to result in a 90% lethality at 24 hr. Control animals were injected intravenously with 0.9% saline. Animals were sacrificed by decapitation and hearts were rapidly removed and perfused with ice-cold saline through the aorta to remove blood. Chilled hearts were trimmed of adipose tissue, large vessels and atria and the ventricles were frozen by compression between brass tongs previously cooled in liquid nitrogen. Frozen tissue was stored in liquid nitrogen until assayed.

Experiments were designed to study myocardial adenylate cyclase

activity at various stages of endotoxemia. The majority of rats subjected to the dose of endotoxin used in this protocol died within 6 hours, therefore rats were killed at early (0.5) or middle (3 hours) points in the time period or when the animals were near death. The terminal stage of endotoxemia is characterized by marked hypoglycemia and lactic acidemia (58) and is considered as that time when the animal loses its righting reflex. Control animals were time-matched such that each time an endotoxic rat succumbed a saline-treated control was sacrificed.

#### Membrane Preparation

Frozen ventricle (approximately 150 mg) was weighed, minced and placed in 10 volumes of ice-cold grinding buffer (5 mM Tris-HCl, 1 mM  $MgCl_2$ , 250 mM sucrose, pH 7.4). The tissue was allowed to thaw then homogenized with two 15-sec bursts with a 15-sec cooling interval, using a Tekmar Tissumizer set at speed 7 with probe generator STD100EN (Tekmar Co., Cincinnati, OH). The homogenate was centrifuged at 48,000 x g for 15 min in a Sorvall RC-5 (Dupont Co., Wilmington, DE). The supernatant was discarded and the pellet resuspended in 20 volumes of suspension buffer (75 mM Tris-HCl, 15mM  $MgCl_2$ , pH 7.53 at 25 ° C). The suspension was centrifuged and resuspended twice more. The final pellet was suspended in 20 vol of the suspension buffer and filtered through four layers of surgical gauze. The final membrane protein concentration was approximately 2.0 mg of protein/ml.

Protein concentration of the membrane suspension was measured by

the Bradford method (13) using bovine serum albumin as the standard.

### Adenylate Cyclase Activity

This procedure was based on the methods of O'Connor et al (100) with some modification. Approximately 75 ug of membrane protein were added to a total volume of 150 ul of 75 mM Tris-HCl (pH 7.4), 8 mM KCl, 7.5 mM theophylline, 0.4 mM dithiothreitol, 0.4 mg/ml bovine serum albumin, 0.7 mM EGTA, 6 mM  $MgCl_2$ , 1 mM ATP, 2.5 mM creatine phosphate, 0.075 mg/ml creatine phosphokinase, and 1.0 mM ascorbic acid in a 1.5 ml polyethylene microcentrifuge tube (Sarstedt, Princeton, NJ). The mixtures were incubated for 20 min at 30° C. In certain assay conditions either 8 mM NaF,  $10^{-4}$  M Gpp(NH)p (guanylyl-imidodiphosphate),  $10^{-4}$  M GTP (guanosine-triphosphate), or  $10^{-4}$  GTP plus  $10^{-9}$  to  $10^{-3}$  M (-) isoproterenol were included. After the incubation period the reaction was terminated by the addition of 700 ul cold 1 mM EDTA, 50 mM sodium acetate (pH 7.2). The mixtures were centrifuged for 10 min at 10,000 x g in an Eppendorf Model 5414 Microfuge (Brinkmann Inst. Co., Westbury, NY) and the supernatant was decanted into 12x75 mm glass tubes and stored at -20° C until assayed for cAMP. Cyclic AMP was measured by radioimmunoassay using a kit obtained from Becton Dickinson Immunodiagnosics (Orangeburg, NY). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

### Statistical Analysis

The maximum stimulated adenylate cyclase activity and the concentration of isoproterenol required to half maximally stimulate

adenylate cyclase activity ( $EC_{50}$ ) for each assay were determined using the ALLFIT program (22). Control and endotoxic values at each stage were compared by unpaired Student's *t* test. When differences in  $EC_{50}$  were observed, lines were also compared by analysis of covariance.



## RESULTS

Various pharmacological probes were employed to stimulate adenylate cyclase activity. Figure 1 shows isoproterenol stimulation of rat heart ventricular membrane adenylate cyclase after 0.5 hours of endotoxycosis. There are no significant differences between baseline values for control and endotoxin treated animals or at any concentration of isoproterenol above  $10^{-8}$  M as compared by unpaired Student's t-test. There are small but significant differences in cyclase activation at  $10^{-10}$  M to  $10^{-8}$  M isoproterenol. In each case studied the control value is greater than the experimental value.

Isoproterenol stimulation of adenylate cyclase activity three hours after endotoxin administration is shown in figure 2. There are no significant differences between basal levels or any degree of isoproterenol stimulation.

At the terminal stage of endotoxycosis, when the rats are in a shock state, the basal levels of adenylate cyclase activity are not different. Mean isoproterenol-stimulated cyclase activity is markedly different from  $10^{-8}$  M to  $10^{-5}$  M however the values reach statistical significance only at a concentration of  $10^{-7}$  M (fig. 3).

In Table 1 the concentration (-log molar) of isoproterenol required to stimulate adenylate cyclase half-maximally ( $EC_{50}$ ) and the

Figure 1.

Effect of isoproterenol on adenylate cyclase activity in membranes derived from ventricular myocardium from control and endotoxin-treated rats 0.5 hours after saline or endotoxin administration. Each point represents the mean  $\pm$  SEM of 4-5 preparation, each from a different animal.

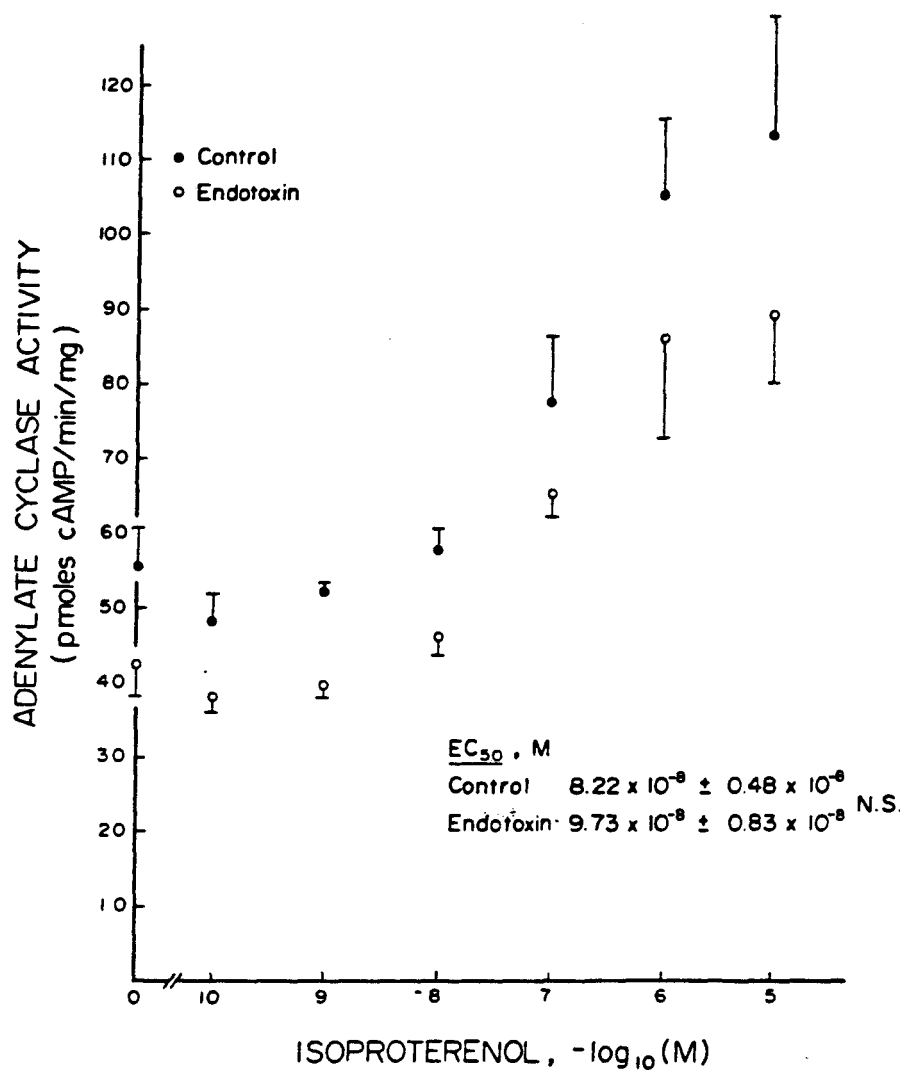


Figure 2.

Effect of isoproterenol on adenylate cyclase activity in membranes derived from ventricular myocardium from control and endotoxin-treated rats 3 hours after administration of saline or endotoxin. Each point represents the mean  $\pm$  SEM from 5-6 preparations, each from a different animal.

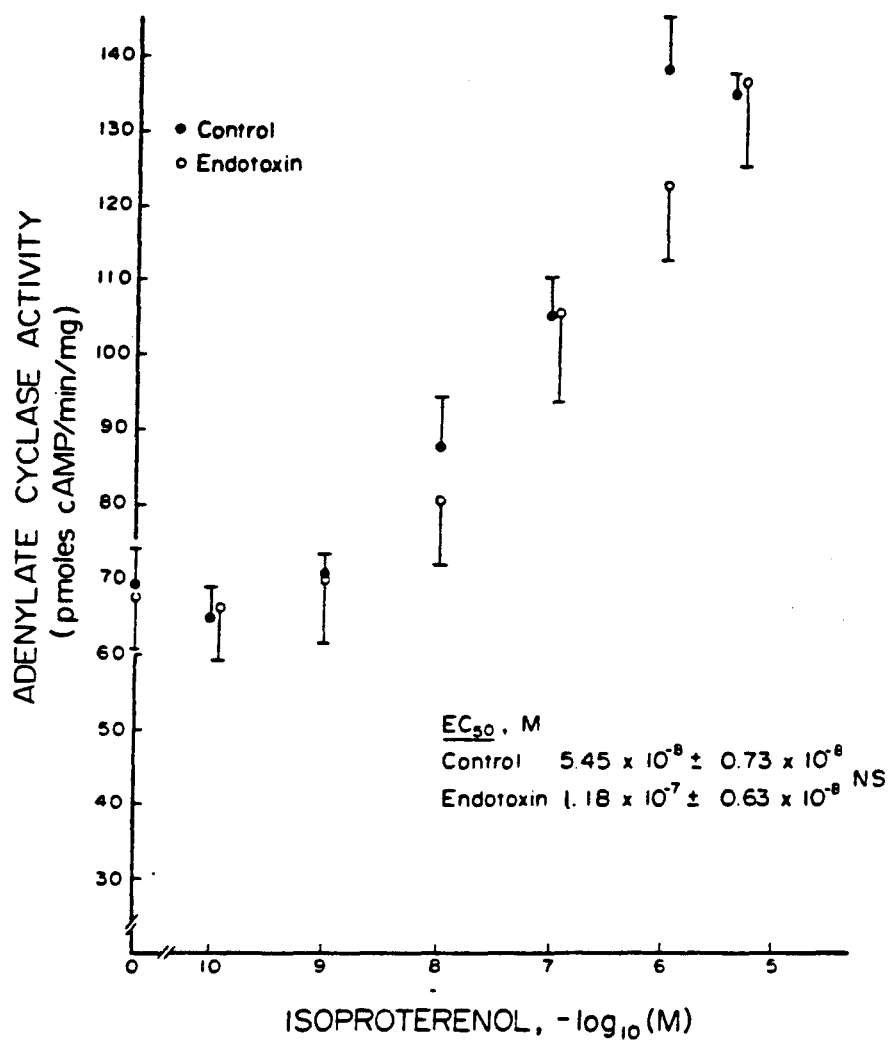


Figure 3.

Effect of isoproterenol on adenylate cyclase activity in membranes derived from ventricular myocardium from control and endotoxin-treated rats at the terminal stage of endotoxicosis. Each point represents the mean  $\pm$  SEM of 5-6 preparations, each from a different animal.

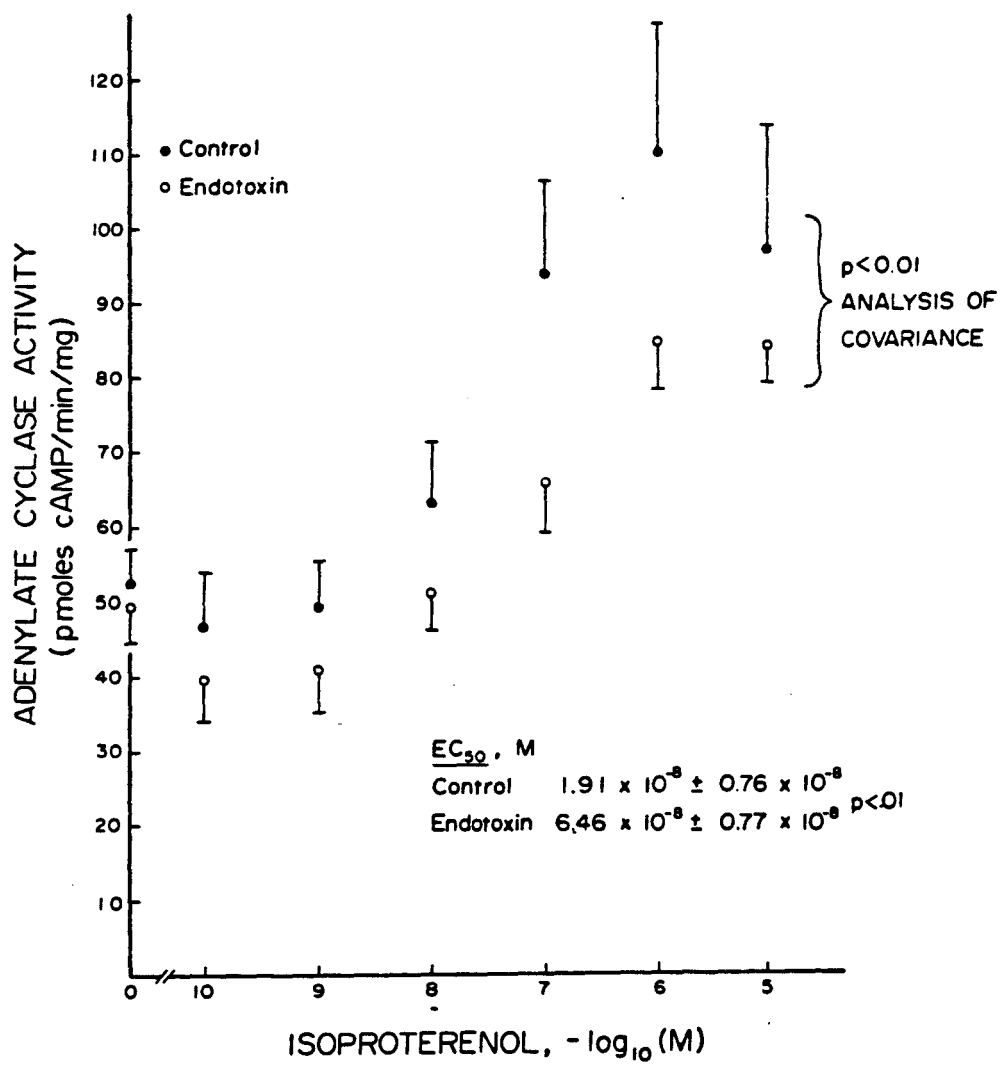


TABLE I. ADENYLATE CYCLASE ACTIVITY

		EC <sub>50</sub> (-log M ISO)	V <sub>max</sub> (pmol cAMP/min/mg)
Stage of endotoxycosis			
0.5 HOURS	Control	7.08±0.32	117.5±18.9
	Endotoxin	7.01±0.08	90.8±10.0
3.0 HOURS	Control	7.26±0.13	141.6±3.5
	Endotoxin	6.93±0.2	143.2±11.0
TERMINAL	Control	7.72±0.11 *	104.3±16.5
	Endotoxin	7.19±0.11	87.0±5.6

\* p&lt;0.05



maximum stimulation ( $V_{\max}$ ) are compared by unpaired t-test at each stage of endotoxiosis. The values presented are means of data from individual experiments. Values for each experiment were generated by the ALLFIT program. The  $EC_{50}$  for the endotoxin group is not significantly different from control at 0.5 or 3 hours, although the  $EC_{50}$  is somewhat greater for both control groups. At these times there is no difference in  $V_{\max}$ . At the terminal stage the  $EC_{50}$  is shifted significantly to the right in the endotoxin animal as compared to control. The values ( $7.19 \pm 0.11$  vs.  $7.72 \pm 0.11$ ,  $-\log$  isoproterenol concentration or  $7.54 \times 10^{-8}$  M vs.  $2.14 \times 10^{-8}$  M) represent a greater than threefold increase in the concentration of isoproterenol necessary to stimulate adenylate cyclase half-maximally in the terminal stages. Since this shift was observed only in the terminal group the data at that stage was also analyzed by analysis of covariance. This method analyzes the linear part of each dose response curve and compares lines with similar slopes. The dose response curves were shown to be significantly different by this method at terminal endotoxiosis. The  $V_{\max}$  is not significantly different in the terminal groups, however the mean values are lower in the endotoxic animal.

Adenylate cyclase was also stimulated in ventricular membranes with NaF or Gpp(NH)p, substances that activate cyclase at some point distal to the receptor, presumably via binding to the guanine-nucleotide regulatory protein. There was no difference in the ability of either NaF (fig. 4) or Gpp(NH)p (fig. 5) to stimulate control or endotoxic membrane cyclase at any stage of endotoxiosis.

Figure 4.

Effect of NaF (7 mM) on adenylate cyclase activity in membranes derived from ventricular myocardium from control and endotoxin treated rats 0.5, and 3 hours after saline or endotoxin administration or at the terminal stage of endotoxiosis. Each bar represents the mean  $\pm$  SEM of 5-6 preparations, each from a different animal.

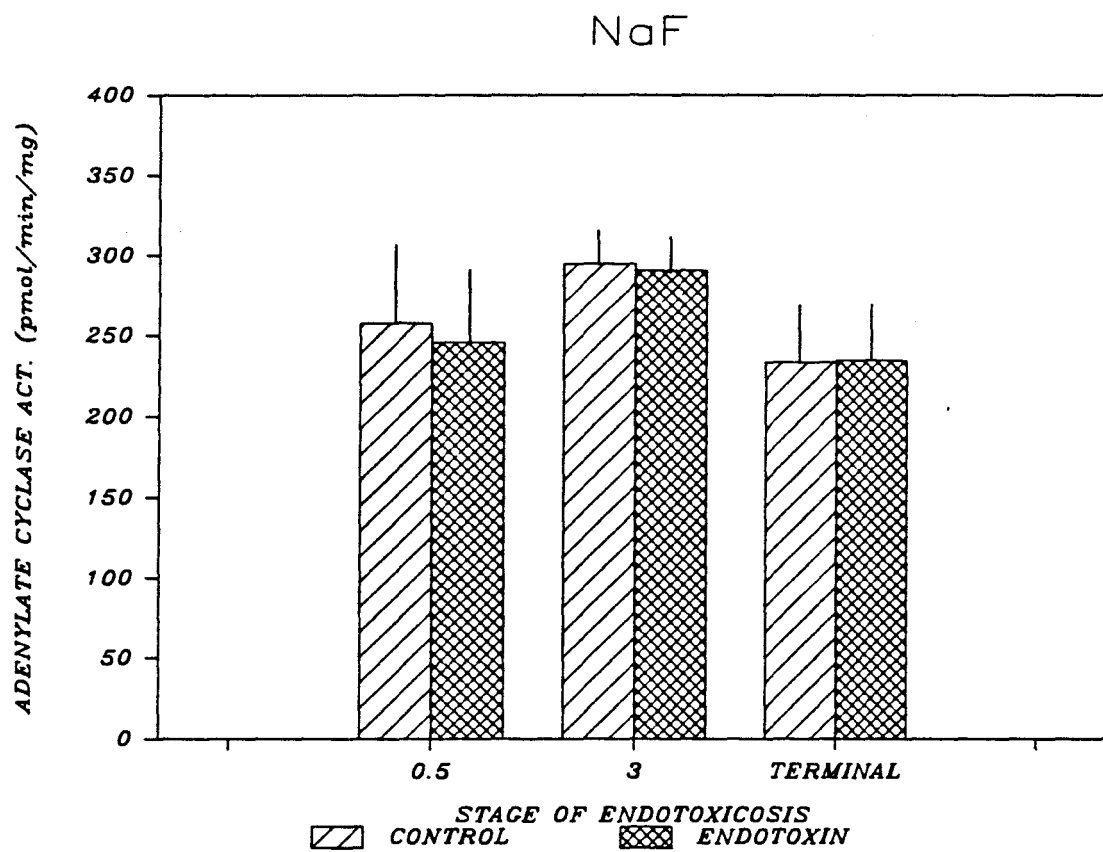
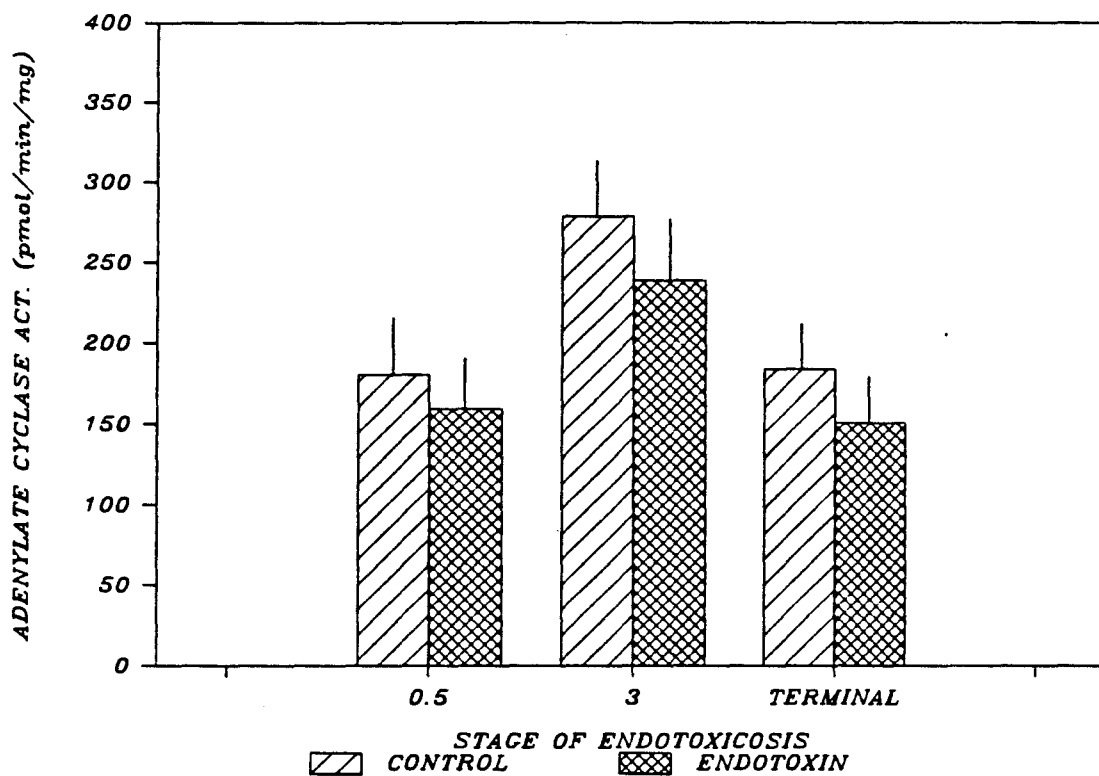


Figure 5.

Effect of guanylylimidodiphosphate (GppNHp) on adenylate cyclase activity in membranes derived from ventricular myocardium from control and endotoxin-treated rats at 0.5 and 3 hours after saline or endotoxin administration or at the terminal stage of endotoxemia. Each bar represents the mean  $\pm$  SEM of 5-6 preparations, each from a different animal.

Gpp(NH)p



## DISCUSSION

The data presented in this study demonstrate that the beta-adrenergic receptor-adenylate cyclase system remains intact in the rat ventricle until the terminal stage of endotoxycosis. During the early stages of endotoxycosis there is no evidence for a lesion at any point in the system, however at the later stage there appears to be modification of at least one component or interaction between the components. It is currently believed that there are three major components of the adenylyte cyclase activating system: the receptor, the catalytic moiety of the enzyme, and the guanine-nucleotide regulatory protein which acts as a transducer between receptor occupancy and enzyme activation (82). An alteration of any component of this system would result in an attenuated production of cAMP. Cyclic AMP is considered the prime mediator of adrenergic stimulation of contractility in the heart (101). If the production of cAMP in response to adrenergic agonists was compromised it would severely limit the ability of the sympathetic nervous system to support myocardial function during stress.

To determine if changes in the catalytic moiety of the enzyme or interaction of the guanine nucleotide regulatory protein and catalytic moiety occurred during the endotoxycosis, membrane preparations were challenged with 8 mM NaF or 0.1 mM Gpp(NH)p. The stimulation of adenylyte cyclase activity by NaF or Gpp(NH)p is a result of binding to the guanine nucleotide regulatory protein. This enhances the association

of the protein with the catalytic moiety and thus catalytic activity is augmented. The concentrations of NaF and Gpp(NH)p used in this study are within the range that would maximally stimulate cyclase activity. Since there was no difference at any time during the endotoxemia between control and endotoxic membrane response to either substance, this suggests that the interaction between the guanine nucleotide regulatory protein and the catalytic moiety is intact. This also implies that the catalytic unit can be maximally activated in the endotoxic tissue. Direct stimulation of the catalytic unit by forskolin might better reflect the state of the enzyme. However, since there were no differences in  $V_{\max}$  between control and endotoxic animals when either NaF or Gpp(NH)p were used, activation by forskolin would not give any further information.

Stimulation of adenylate cyclase activity with adrenergic agonists involves all the components of the system (127). To assess agonist activation of adenylate cyclase, membrane preparations were incubated with varying concentrations of isoproterenol. At the terminal stage of endotoxemia the dose response curve to isoproterenol is shifted to the right without any significant loss in maximum stimulation. This suggests that some change in the receptor-agonist interaction or receptor-guanine nucleotide regulatory protein association has occurred. Receptor number and affinity for ligand have been observed to decrease in various physiological or pathophysiological situations (45). The possibility of one or both of these happening in the heart during endotoxemia cannot be discounted. Liu and Ghosh (85)

have reported that endotoxin administration in vivo (1 mg/kg) decreased both the binding affinity of radiolabeled antagonist and the density of beta-adrenergic receptors in dog liver plasma membranes at 2 hours after the injection. Isoproterenol-stimulated adenylate cyclase was also inhibited in the same tissues (32). Fluoride and guanine-nucleotide activation were similarly depressed in this tissue. In addition, these effects of endotoxin on receptors and adenylate cyclase were also observed in in vitro experiments.

The present data suggest that the coupling of receptor to adenylate cyclase may be altered, regardless of whether there are alterations in the number of receptors or receptor affinity for antagonists in the myocardium. The interaction of beta-adrenergic receptors with adenylate cyclase may be dependent upon the fluidity of membrane of which they are a part. Hirata et al (60) have shown that beta-receptor agonists increase phospholipid methylation, such that phosphatidylethanolamine is converted to phosphatidylcholine, and membrane fluidity is enhanced. This is associated with an increased coupling of receptor with cyclase and production of cAMP. Furthermore Limas (79) has shown phospholipid methylation to regulate the number of beta-adrenergic receptors in myocardial membranes. Liu and his colleagues have studied membrane fluidity of myocardial and hepatic membranes exposed to endotoxin in vivo or in vitro (84,86). Their data shows a increase in phospholipase A activity in both endotoxic membranes as compared to their respective controls. This is associated with an increase in phosphatidylcholine hydrolysis and decreased membrane



fluidity. It appears that the endotoxin may directly stimulate phospholipase activity in these membranes. The decrease in fluidity is thought to be at least partially responsible for decreased adenylate cyclase activity in liver plasma membranes. In the present study alterations in membrane fluidity would have to be considered as a factor in the decreased sensitivity of cyclase activation in the latter stages of endotoxycosis.

The myocardium is known to lose responsiveness to catecholamines during exposure to partial or full agonists. This tachyphylaxis or desensitization may be the result of receptor down regulation or uncoupling of the receptor from adenylate cyclase (127). Short term exposure (a few hours or less) of myocardial tissue to elevated levels of catecholamines in vivo or in vitro have been shown to attenuate contractile and adenylate cyclase responses to catecholamines (90,138) without changes in receptor number or affinity. Chronic catecholamine administration (days) in vivo is associated with down-regulation of receptors as well attenuated cyclase response to agonists (18).

Jones and Romano (63) have reported plasma concentrations of norepinephrine and epinephrine to increase 40 and 100 times, respectively, by the terminal stages of endotoxycosis in rats receiving the same dose of endotoxin as the present study. The supranormal levels of norepinephrine are apparently due to increased nerve stimulated release (105). The data of Kopin et al (67) suggest that in conditions of nerve-stimulated release the concentration of norepinephrine at the

post-synaptic membrane will be considerably higher than that measured in the plasma. Therefore plasma levels in the Jones and Romano (63) study underestimate the levels at the receptor. While these concentrations represent a significant increase above baseline levels the concentrations at the beta-receptor (approximately  $10^{-7}$  M), as calculated using the Kopin work, are not of the same magnitude as most of the concentrations used in in vitro or infusion studies that have observed the desensitization phenomenon (43). In this respect the effect of endogenous catecholamine elevations may not be to the same extent or follow the same time course as those studies in which desensitization has been demonstrated with exogenous catecholamines. In the present study agonist concentration at the beta-receptors may be increased enough for the duration of the endotoxemia (5 hours) to cause a modest desensitization of these receptors in the shocked rat myocardium and this could account for the rightward shift in the adenylate cyclase dose response curve to isoproterenol. If the total number of receptors is not changed this could account for similar maximum responses in both tissues. Although there is no significant difference in maximum cyclase activation values at the terminal stage, the mean values of the endotoxic group are much lower. It is possible that the difference could reach significance if the time of the myocardial exposure to catecholamines was lengthened.

The mechanism of receptor uncoupling during agonist-specific desensitization is yet to be determined, however decreasing membrane fluidity has been suggested as a possible mechanism. Quinacrine, a

phospholipase A inhibitor, has been observed to attenuate the loss of receptors during chronic isoproterenol administration (131). Others have found an altered phosphorylated form of the receptor after chronic exposure to catecholamines that may account for problems of coupling to the cyclase (124). Recently, Bobik and Little (9) have found that heart cells treated with phosphodiesterase inhibitors manifest signs of desensitization which suggests that receptor alterations are cAMP mediated.

Regardless of the mechanism contributing to decreased production of cAMP, data presented here suggest that the decreased functional responsiveness to catecholamines during endotoxiosis observed in a number of laboratories is based on alterations at the cellular level. Myocardial dysfunction during endotoxiosis appears to be the result of a number of abnormalities. Coronary hypoperfusion, intracardiac ionic imbalances, depression in the ability of the sarcoplasmic reticulum to sequester calcium and depression of contractile protein function are all believed to contribute to the failure (49). While the loss of adrenergic responsiveness is not likely to be the sole cause of the myocardial failure of endotoxiosis, the loss of adrenergic support would exacerbate the decline in myocardial function (40,118). Protection of beta-adrenergic receptors may be of therapeutic value during episodes of clinical sepsis.

In conclusion, the loss of sensitivity of adenylate cyclase activation to isoproterenol stimulation in the endotoxic rat heart

appears to be an uncoupling event. However to better understand the mechanisms involved, future experiments should evaluate the binding characteristics of beta-adrenergic agonists and antagonists in the heart at various stages of endotoxycosis.

## CHAPTER V

### CHARACTERISTICS OF MYOCARDIAL BETA-ADRENERGIC RECEPTORS DURING ENDOTOXICOSIS IN THE RAT

## INTRODUCTION

Sympathetic nervous system activity is known to support the cardiovascular and metabolic adjustments to stress. However prolonged periods of elevated sympathetic activity may be detrimental and contribute to the pathogenesis of the stress state. Sepsis and endotoxycosis are conditions in which augmented sympathetic nervous system activity is at the same time both necessary for and deleterious to cardiovascular regulation of homeostasis. It appears that support is extended mainly through the beta-adrenergic limb of the system and not the alpha-adrenergic component. Beta-adrenergic blockade during endotoxycosis has been shown to increase lethality in rats (31), whereas alpha-adrenergic blockade or selective denervation protected against the lethality of endotoxic shock in both rats and dogs (31,77). Beta-adrenergic receptor stimulation has been shown to be particularly important to maintaining myocardial performance during sepsis or endotoxycosis since blockade of myocardial beta-receptors severely compromises myocardial function (40,16).

Studies from a number of laboratories have determined that the response to catecholamines is decreased in the latter stages of endotoxycosis (1,6,107). With the apparent myocardial requirement for sympathetic nervous system support of the myocardium during endotoxycosis it is significant that the myocardial response to catecholamine stimulation is diminished. There has been no attempt in

any previous study to determine the mechanism of the attenuated responsiveness. Possible explanations for the decreased responsiveness are: 1) inability of the heart muscle to respond to inotropic stimuli of any type; 2) high existing levels of plasma catecholamines, which could mask the response to exogenous agents (in vivo studies); or 3) alterations of the beta-adrenergic effector system.

It has previously been reported that isoproterenol-stimulated CAMP accumulation in rat ventricular slices was attenuated in the terminal stage of endotoxiosis (114) and that there is a decreased sensitivity of adenylate cyclase to isoproterenol stimulation at the same time (115). These changes do not appear to be due to a modification of the enzyme itself or to deterioration of activation via the guanine nucleotide regulatory protein. This leaves the receptor as the possible point of the lesion. Therefore the purpose of this study was to assess myocardial beta-receptor characteristics at various stages of endotoxiosis in the rat using radioligand binding techniques.

## METHODS

Animal Model

Healthy male Holtzman rats (Holtzman Company, Madison, WI) weighing between 300 and 400 g were used in this study. Rats were housed in wire-mesh cages at 22° C ambient temperature with a 12-hr light-dark cycle and provided with food (Wayne Lab Blox) and water ad libitum. Rats were kept in this manner for at least one week prior to use in experimental protocols.

Endotoxin (Difco Laboratories, Detroit, MI) was purchased as a single batch (703252) of Boivin lipopolysaccharide from Salmonella enteritidis. The endotoxin injectate was prepared fresh daily in 0.9% saline (5 mg/ml). Rats were lightly anesthetized with ether and injected with endotoxin (16.7 mg/kg) intravenously via the dorsal vein of the penis. Previous experiments had shown this dose of endotoxin to result in a 90% lethality at 24 hr. Control animals were injected intravenously with 0.9% saline. Animals were sacrificed by decapitation 0.5 and 3 hours after endotoxin administration or in the terminal stages of endotoxiosis as determined by loss of the righting reflex. Each time an endotoxic animal was killed a control animal was also sacrificed. Hearts were rapidly removed and perfused with ice-cold saline through the aorta to remove blood. Chilled hearts were trimmed of adipose tissue, large vessels and atria and the ventricles were frozen by compression between brass clamps previously cooled in liquid nitrogen. Frozen tissue was



stored in liquid nitrogen until assayed.

### Membrane Preparation

Suitable cardiac membrane preparations were prepared as previously described by Baker et al (3) with minor modification. Frozen ventricles were weighed, minced and placed in 10 ml of ice-cold grinding buffer (5 mM Tris-HCl, 1 mM  $MgCl_2$ , 250 mM sucrose, pH 7.4). The tissue was allowed to thaw then homogenized with two 15-sec bursts with a 15-sec cooling interval, using a Tekmar Tissumizer set at speed 7.5 with probe generator STD100EN (Tekmar Co., Cincinnati, OH). The homogenizer was rinsed with 5 ml of grinding buffer and this was added to the homogenate. An equal volume (15 ml) of cold 1 M KCl was added to the homogenate and the mixture was continuously stirred on ice for 15 min. The homogenate was centrifuged at  $48,000 \times g$  for 15 min in a Sorvall RC-5 (Dupont Co., Wilmington, DE). The supernatant was discarded and the pellet resuspended in 25 volumes of suspension buffer (75 mM Tris-HCl, 15mM  $MgCl_2$ , pH 7.53 at  $25^\circ C$ ). The suspension was centrifuged and resuspended twice more. The final pellet was suspended in 25 vol of the suspension buffer and filtered through four layers of surgical gauze. The final membrane concentration was 0.8-1.6 mg/ml.

Protein concentration of the membrane suspension was measured by the Bradford method (13) using bovine serum albumin as the standard. DHA was prepared in 0.1 mM ascorbic acid. Isoproterenol was prepared in 1 mM HCl/0.1 mM ascorbic acid.

### Binding Experiments

Binding assays were based on the methods of Lurie et al (88) with some modification. In the saturation experiments 250  $\mu$ l aliquots of the membrane preparation were incubated with increasing concentrations of ( $^3$ -H)-dihydroalprenolol (DHA, sp. act. 35.6 Ci/mmol) with or without (-) isoproterenol  $10^{-3}$  M in a final volume of 500  $\mu$ l of incubation buffer (75 mM Tris-HCl, 15 mM  $\text{MgCl}_2$ , 1 mM ascorbic acid, pH 7.4); the final concentration of DHA ranged from 0.3 to 17.0 nM. The mixtures were incubated at 30° C for 30 min. At the end of the incubation period binding was terminated by adding 4 ml of ice-cold buffer (75 mM Tris-HCl, 15 mM  $\text{MgCl}_2$ , pH 7.4) to the mixtures then filtering on Whatman GF/C filter papers using a Brandel Cell Harvester (Gaithersburg, MD). Each filter was washed with 6-9 ml of buffer. The filters were placed in 5 ml mini-scintillation vials (RPI, Mt. Prospect, IL) and allowed to dry. The vials were filled with 3.5 ml of scintillation fluid (3a70, RPI) and the radioactivity was counted in a Packard Tri-Carb 460 (Packard Instrument Co., Downers Grove, IL). Specific binding was determined by subtracting DHA binding in the presence of  $10^{-3}$  M (-) isoproterenol from DHA binding in the absence of isoproterenol. Specific binding was generally 60-65% of total binding at the  $K_d$  for DHA.

Saturation data were analyzed using non-linear regression with weighting. The use of this method was previously reported by Lurie et al (88) and a listing of the program was graciously supplied by Dr. Michael R. Bristow.

In competition studies aliquots of membrane protein were incubated with a single concentration of DHA (2-4 nM) and 15 increasing concentrations of (-) isoproterenol ( $10^{-10}$  M to  $10^{-3}$  M). The assay was then performed exactly as the saturation experiments.

Competition data were analyzed by two methods. The concentration of isoproterenol required to inhibit 50% of DHA binding ( $IC_{50}$ ) and the slope factor (pseudo-Hill coefficient) of the competition curve were derived from a four-parameter logistic equation, the ALLFIT program of DeLean et al (22). Where differences in the slope factors of the competition curves existed between endotoxic and control membranes the curves were modelled to a one or two state fit using LIGAND, the non-linear curve modelling program of Munson and Rodbard (98).

( $^3H$ ) dihydroalprenolol was purchased from New England Nuclear (Boston, MA). All other reagents were purchased from Sigma Chemical co. (St. Louis, MO).

### Statistical Analysis

Student's t test or a one way analysis of variance were used, when appropriate, for the statistical analysis of the data. Determining goodness of fit for a binding model was based on the "extra sum of squares" principle (98). A two state model was accepted only if it significantly improved the fit over a one state model.

## RESULTS

### Saturation Binding Assays

General beta-adrenergic receptor characteristics were determined by performing saturation binding assays using the antagonist ( $^3\text{H}$ )dihydroalprenolol. Figures 1, 2 and 3 depict binding data from grouped experiments 0.5 and 3.0 hours after endotoxin or saline administration or during the terminal stages of endotoxemia. Mean specific binding at each concentration of DHA for control and endotoxin-treated animals are shown on each graph. The inset of each graph contains average data represented as Scatchard plots at each stage.

( $^3\text{H}$ )DHA binding data are summarized in Table I. Maximum DHA binding ( $B_{\text{max}}$ ) and the dissociation constant ( $K_d$ ) of DHA were determined by non-linear regression analysis and compared at each stage of endotoxemia. There were no significant differences in either parameter at any time during the endotoxemia as determined by unpaired student's t test. Furthermore there were no differences among the control groups or the endotoxin groups. Specific binding averaged  $63.7\% \pm 2.3\%$  in saline treated hearts and  $60.4\% \pm 1.9\%$  in endotoxin treated hearts.

### Competition Binding Experiments

Agonist binding characteristics were determined at each stage by performing isoproterenol-DHA competition assays. The mean values for

Figure 1.

Specific binding of 3-dihydroalprenolol (DHA) to ventricular or endotoxin administration. Each point represents the mean  $\pm$  SEM of 5-6 preparations, each from a different animal. Inset shows Scatchard plot of DHA binding, determined from mean values.

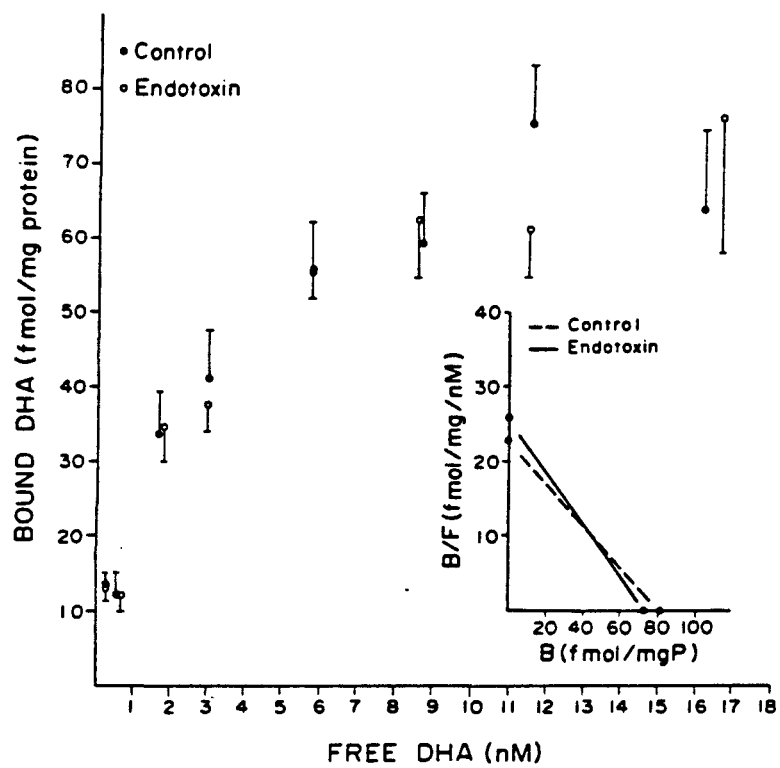


Figure 2.

Specific binding of  $^3\text{H}$ -dihydroalprenolol (DHA) to ventricular membranes from control and endotoxin-treated rats 3 hours after saline or endotoxin administration. Each point represents the mean  $\pm$  SEM of 4-5 preparations, each from a different animal. Inset shows Scatchard plot of DHA binding determined from mean values.

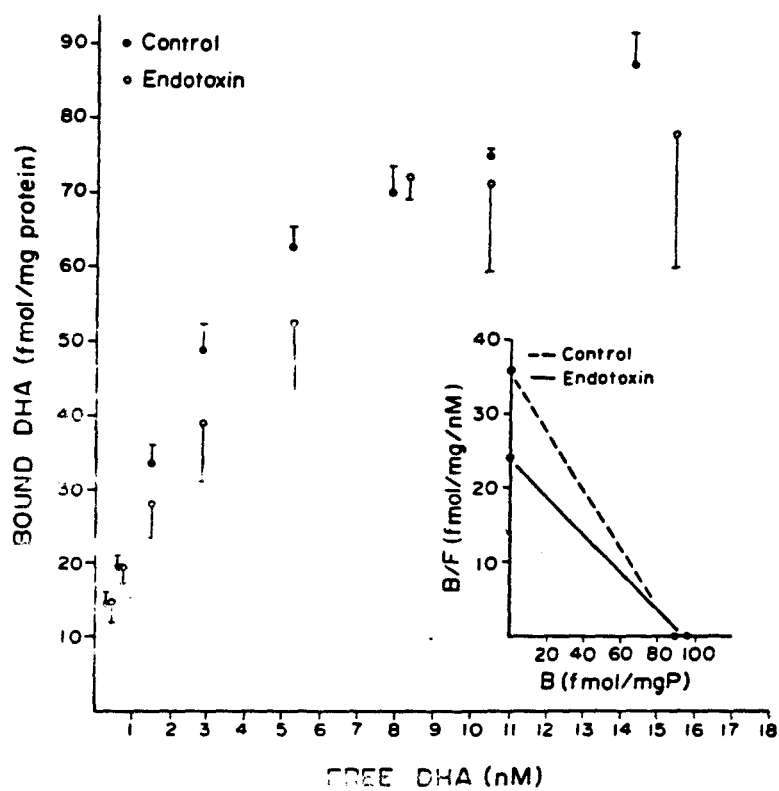




Figure 3.

Specific  $^3\text{H}$ -dihydroalprenolol binding to ventricular membranes from control and endotoxin-treated animals at the terminal stage of endotoxycosis. Each point represents the mean  $\pm$  from 8-10 preparations, each from a different animal. Inset shows Scatchard plot for DHA binding determined from mean values.

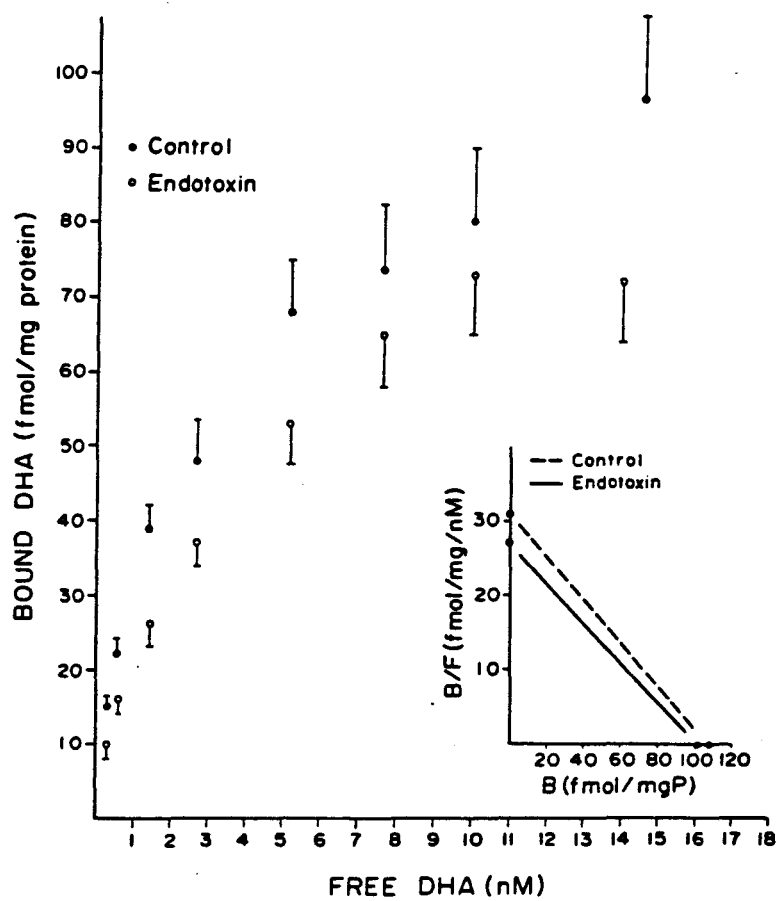


TABLE I.  $^3\text{H}$ -DIHYDROALPRENOLOL BINDING CHARACTERISTICS

		$K_d$ (nM)	$B_{\max}$ (fmol/mg)
Stage of endotoxycosis			
0.5 HOURS	Control	$3.53 \pm 1.01$	$82.0 \pm 9.8$
	Endotoxin	$2.79 \pm 0.89$	$73.0 \pm 7.9$
3.0 HOURS	Control	$2.51 \pm 0.39$	$90.4 \pm 2.3$
	Endotoxin	$3.99 \pm 0.69$	$95.9 \pm 15.9$
TERMINAL	Control	$3.46 \pm 0.83$	$108.4 \pm 16.7$
	Endotoxin	$3.78 \pm 0.65$	$101.7 \pm 15.3$

data derived from these assays is displayed in Table II. The dose of isoproterenol required to inhibit 50% of DHA binding ( $IC_{50}$ ) was not significantly different between control and endotoxin groups at any time.

The slope factors for each competition curve were also determined. This value is essentially the same as the Hill coefficient. It is called a pseudo-Hill coefficient because the binding of the agonist is indirectly assessed by virtue of its displacement of radiolabeled antagonist. When the binding reaction follows the principles of mass action the slope factor is 1.0. A slope factor significantly different from 1.0 implies that the ligand receptor reaction is more complex than simple mass action. A slope factor less than one is commonly the result of heterogeneity of binding sites. The slope factors at 0.5 and 3 hours are not significantly different and are less than 1.0. At the terminal stage of endotoxiosis the slope factors for control and endotoxic isoproterenol binding are significantly different. The control is significantly less than 1.0 whereas the endotoxin value approaches 1.0.

Figure 4 depicts representative isoproterenol competition curves at the terminal stage of endotoxiosis. The curve for the control tissue is shallow and has a slope factor of 0.54. The curve for the endotoxic tissue is of normal steepness and has a slope factor close to 1.0. A trailing foot is noticeable at the high dose range. The data in Table III are the average values for isoproterenol binding at the terminal

TABLE II. ISOPROTERENOL BINDING CHARACTERISTICS

Stage of endotoxycosis		<u>IC<sub>50</sub>(-log M ISO)</u>	<u>SLOPE</u>
0.5 HOURS	Control	7.65±0.13	0.78±0.16
	Endotoxin	7.84±0.19	0.61±0.03
3.0 HOURS	Control	7.86±0.13	0.64±0.07
	Endotoxin	7.44±0.17	0.71±0.07
TERMINAL	Control	7.06±0.16	0.60±0.06
	Endotoxin	7.27±0.11	0.91±0.12

\* p&lt;0.05

Figure 4.

$^3\text{H}$ -dihydroalprenol competition curves for isoproterenol in ventricular membranes from control and endotoxin-treated rats at the terminal stage of endotoxycosis. The data points are the means of duplicate determinations in representative experiments.

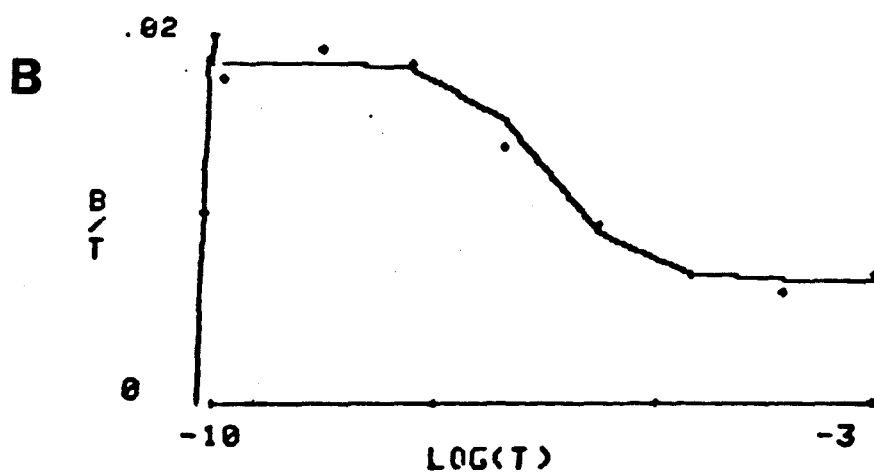
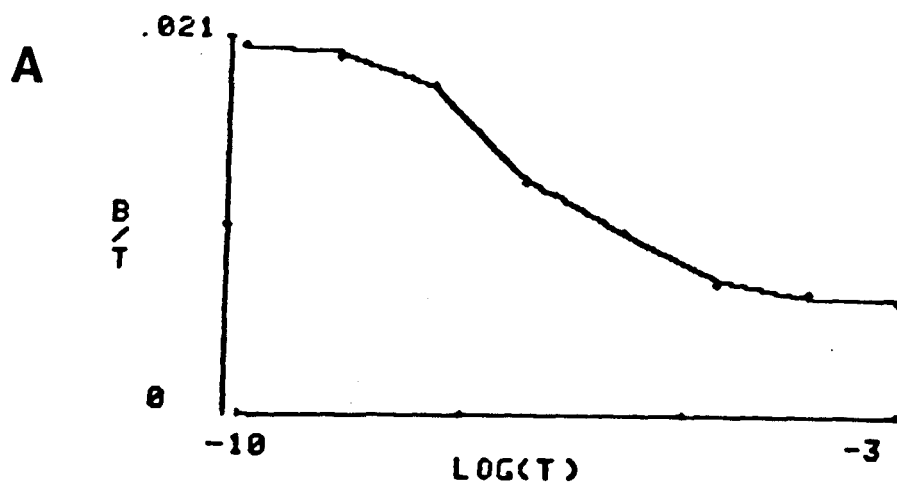


TABLE III. ISOPROTERENOL BINDING PARAMETERS  
IN TERMINAL ENDOTOXICOSIS

	<u>CONTROL</u>	<u>ENDOTOXIN</u>
$K_1$ (nM)	4.6 $\pm$ 0.8	33.9 $\pm$ 14.5
$K_2$ (nM)	1883 $\pm$ 827	---
$K_2/K_1$	466 $\pm$ 211	---
$R_1$ (%)	60.3 $\pm$ 5.2	100
$R_2$ (%)	39.7 $\pm$ 5.2	---



stages of shock as determined by computerized non-linear curve fitting methods (98). The curves for the control membranes are best explained by two affinity states of the receptor, 60% of the total receptor population are of high affinity ( $R_1$ ) with a dissociation constant ( $K_1$ ) of  $4.6 \pm 0.8$  nM; 40% of the receptors are of low affinity ( $R_2$ ) with a dissociation constant of  $1883 \pm 827$  nM. The ratio of the dissociation constants for high and low affinity states ( $K_2/K_1$ ) was  $466 \pm 211$ . The curves for the membranes from endotoxin treated animals modeled best to a single population of receptors with relatively low affinity ( $33.9 \pm 14.5$  nM) for the agonist. The existence of two heterogeneous states was no longer apparent at the terminal stages of endotoxemia.

## DISCUSSION

Previous data from our laboratory has indicated a decrease in sensitivity of adenylate cyclase activation to the beta-adrenergic agonist, isoproterenol at the terminal stages of endotoxiosis (115). It was proposed that this was due to a problem at the receptor level and not with the enzyme, itself. The data from the present study support the hypothesis that the changes at terminal endotoxiosis are due to a coupling problem between the agonist-receptor complex and the catalytic unit of adenylate cyclase.

It is currently believed that beta-adrenergic agonists promote the formation of a ternary complex, composed of the agonist, the receptor and the guanine nucleotide regulatory protein (N protein or N). Formation of this complex results in a high affinity state of the receptor. The ternary complex is transient and is destabilized by GTP. When the agonist-receptor complex is no longer associated with the guanine nucleotide regulatory protein it reverts to a low-affinity state. GTP binding to the alpha subunit of the N protein induces a conformational change such that the alpha and beta subunits dissociate and GDP is released. The N-GTP unit complexes with the catalytic moiety of adenylate cyclase, thereby activating it and initiating cAMP production. Cyclic AMP then mediates the events that result in enhanced inotropism, primarily via phosphorylation of intracellular proteins. If an antagonist is added to the system rather than an agonist the chain of

events is blocked because there is no impetus to form the crucial ternary complex (23,82,127).

Competition curves for agonists in the absence of guanine nucleotides are shallow with slope factors less than 1.0. Computer analysis of these curves reveals that the receptor exists in two affinity states, one high and one low. The high affinity state presumably reflects receptors that are complexed with agonist and N protein while the low affinity state reflects receptor-agonist complexes that have dissociated from N. If high concentrations of guanine nucleotides are added the competition curve steepens and the slope factor approaches 1. Analysis shows all the receptors to be in a low affinity state as a result of destabilization of the ternary complex. Competition curves for antagonists are also steep with slope factors of 1.0, which reflects the inability of antagonists to induce the formation of a ternary complex. All of the receptors appear to be in a low affinity state (65).

The phenomenon of tachyphylaxis or desensitization has been recognized for many years and denotes a state of diminished responsiveness to an agonist, drug or hormone after prior exposure to the substance (127). Rats receiving doses of endotoxin comparable to those used in this study have exhibited very high levels of sympathetic nerve activity and plasma catecholamines (63). The concentrations of catecholamines in the vicinity of the receptors may approach those necessary to initiate desensitization within the time course of the

endotoxycosis. Agonist-induced desensitization has been shown to be both time and concentration dependent in vitro (96). The characteristics of desensitization in vivo have not been defined as well although similar mechanisms may be involved. It is certainly possible that the receptor changes seen in this study may be due to catecholamine-induced desensitization.

The phenomena of receptor-cyclase uncoupling and receptor down regulation are thought to be the mechanisms responsible for the desensitization observed after chronic exposure to agonist. In the present experiments we examined the possibility that either or both mechanisms occur during endotoxycosis. DHA binding parameters,  $K_d$  and  $B_{max}$ , do not change at any stage of endotoxycosis. This suggests that there are no gross structural changes in the receptor resulting from the endotoxycosis. Recent studies (46,125,130) have shown that after exposure to agonists beta-adrenergic receptors are internalized in some tissues. These receptors may be recovered in light vesicles which are sedimented during centrifugation. The vesicles sediment at centrifugation forces that vary with the particular tissue (43). Thus if receptors are measured only in the particulate fraction of desensitized tissue, it would appear that receptors were lost, when in fact they were in vesicles. It appears that no other component of the receptor-cyclase system is sequestered with receptor and thus the receptors are not functional when vesicularized (125,134), but they maintain their integrity (128). Vesicular receptors bind lipophilic antagonists with the same affinity as native receptors since antagonist binding is

independent of N protein interactions (47).

Recently, Limas and Limas (80) have described beta-receptor internalization in rat myocardial membranes after prolonged exposure to isoproterenol. The "lost" receptors were recovered in the cytosolic fraction. The apparent lack of change in receptor number in the endotoxic tissue in the present study suggests that either all the receptors are present at the membrane surface, with no down regulation, or that internalized receptors as well as surface receptors are being identified. We have yet to determine if cytosolic receptors are sedimented under the conditions of the assay as performed in the present study. Harden (43) has presented a model for the cellular processing of beta-adrenergic receptors during catecholamine-specific desensitization that differentiates between internalized but detectable receptors and receptors that are lost due to degradation or modification. It is apparent that there is no loss of total receptors in the endotoxic tissues.

Early in the experimental period endotoxin or the mediators of endotoxiosis do not appear to affect agonist binding to receptors as determined by agonist competition curves. Computer analysis showed no difference in slope factors or  $IC_{50}$ . At the terminal stage of endotoxiosis there is no difference in  $IC_{50}$ , but there is clearly a difference in slope factors between the control and endotoxic group ( $0.60 \pm 0.06$  vs.  $0.91 \pm 0.12$ ). When computer analyzed, the curves from control membranes model to a two state fit, one high-affinity state and

one low affinity state, which corresponds well to the current theory. The curves from terminal endotoxic ventricular membranes model to a one state fit, the affinity of the receptor being somewhat less than the high affinity state of the control but considerably greater than the low affinity state. The total loss of the low affinity site is somewhat puzzling. The slope factor, near one, suggests one homogeneous site, yet it might be expected from data analyzed in other desensitized tissues that there would still be two state binding. The high affinity site of frog erythrocytes has been shown to decrease significantly in affinity and number after exposure to agonist, whereas the low affinity site has smaller changes in absolute number and affinity under the same conditions (65). One possible explanation of the present data is that the number of low affinity sites decreases to a such a degree that the computer analysis is not capable of resolving the binding into two states (24). Another possibility is that there is an alteration in dissociation of the ternary complex such that the formation of the low affinity state is modified. This could occur if GTP binding to the N-protein is compromised. Alternatively, some of the receptors from the endotoxic membrane may be in light vesicles. These receptors have been reported to exhibit only low affinity binding for isoproterenol that is not affected by GTP (44).

The lack of change in the  $IC_{50}$  for isoproterenol may be due to the fact that almost half of the receptors in the control membranes are in a very low affinity state and all (or the majority) of the receptors in the endotoxic membranes have an affinity between the high and low

affinity states of the control membranes. Alternatively, any differences in  $IC_{50}$  may be masked by the variability of the  $^3H$ -DHA concentration used in the competition assays (135). Regardless of this apparent discrepancy, the shift to a lower affinity state in the endotoxic membrane suggests some alteration in the ability of the receptor-agonist complex to associate or couple with the N protein. This decrease in receptor affinity for isoproterenol would then appear to account for the rightward shift in the adenylate cyclase dose response curve (115) in ventricular membranes from terminal endotoxic rats.

Decreases in membrane fluidity have been implicated as being responsible for problems with receptor-cyclase coupling. Mallorga et al (89) have noted increased phosphatidylcholine degradation in rat C6 astrocytoma cells exposed to isoproterenol for 2 hours, which was associated with increased phospholipase A activity. Treatment of rats with quinacrine, a phospholipase A inhibitor, prevented desensitization of cardiac beta-adrenergic receptors due to immobilization stress or isoproterenol administration (131). Liu and colleagues have reported increased phospholipase A activity in heart homogenates from dogs treated with endotoxin for three hours (84). More recently they have observed an endotoxin-induced decrease in canine myocardial phospholipid methylation as assessed by methyltransferase activity (87). They suggest that these changes modify the myocardial membrane-lipid profile such that fluidity is decreased. An increase in the viscosity of the membrane in the area of the receptor may inhibit lateral movement in the membrane. This in turn can compromise coupling

of the receptor with the guanine nucleotide regulatory protein and adenylate cyclase.

In avian erythrocytes and certain cultured cell lines the evidence suggests that a change in receptor structure may be responsible for decreased coupling of desensitization. Stadel et al (124) have shown that catecholamine-induced desensitization of adenylate cyclase in turkey erythrocytes correlates with stable modification of the beta-adrenergic receptor and is associated with agonist-promoted phosphorylation of beta-receptor peptides. Additionally, the phosphorylation may be cAMP mediated (123). Bobik and Little (9) have presented data from chick-heart studies that is compatible with the hypothesis that beta-adrenoceptor desensitization could be due cAMP-dependent phosphorylation of the receptor. They found that incubation of cultured chick embryo ventricular cells with phosphodiesterase inhibitors attenuated isoproterenol-stimulation of CAMP production. Desensitization in rat heart may also be due to phosphorylation since it was prevented by cordycepin, a nonspecific phosphorylation inhibitor (81). Sympathetic nerve activity in the rat increases early in endotoxiosis and is maintained at elevated levels for at least 6 hours (63). The cAMP generated in response to adrenergic stimulation of beta-receptors early in endotoxiosis may be influential in initiating the desensitization process in rat myocardial membranes.

It is not known whether the alterations in receptors and the decreased sensitivity of adenylate cyclase to adrenergic stimulation



(115) that occur during endotoxiosis are due 1) directly to the endotoxin, 2) to a mediator produced in endotoxiosis or, 3) to elevated sympathoadrenal activity. The direct effects of endotoxin on in vitro canine hepatic membrane preparations (32,85) may not be present in myocardial tissue, which is much different from hepatic tissue in structure, function and metabolism. Preliminary experiments designed to protect beta-adrenergic receptors from excessive stimulation during lethal endotoxiosis, have been fruitless to this time. Administration of atenolol, a cardioselective beta-receptor antagonist, along with endotoxin exacerbates the effects of endotoxin and the time to death is reduced (unpublished observations). This data does not answer any questions about the role of catecholamines in the myocardial desensitization but it does stress the importance of sympathetic activity for support of the myocardium during endotoxiosis. Future experiments should concentrate on the stimulus for desensitization in the endotoxic myocardium and the underlying mechanisms.

The alterations in the myocardial beta-adrenergic receptor during endotoxiosis appear modest, but the significance of these changes is considerable. During sepsis or endotoxiosis the myocardium is undergoing a progressive deterioration (58). Initially, sympathetic nervous system activity augments and supports myocardial performance. This becomes increasingly important as myocardial function degenerates. However, the myocardial response to adrenergic stimulation is also deteriorating (1,6,107) and at the same time myocardial sympathetic nerve endings are becoming depleted of norepinephrine (104). Thus the

homeostatic mechanisms that were supporting cardiovascular function are exhausted and the conditions are set for circulatory collapse.

## CHAPTER VI

### INTEGRATED DISCUSSION

Sympathoadrenal-myocardial effector mechanisms important during endotoxycosis were studied in the three projects of this dissertation. The primary goal of these studies was to characterize the cellular mechanisms involved in the myocardial response to catecholamines in the endotoxic state as compared to normal.

The first project examined cAMP levels in the rat ventricle in situ during a 6 hour period of endotoxycosis. It was found that cAMP tissue levels were depressed early in endotoxycosis, return to normal at the intermediate stages, and were again depressed at the terminal stages of endotoxycosis. These in situ levels of cAMP are probably not basal levels since plasma concentrations of catecholamines rise dramatically during decapitation (26). Stimulation of ventricular cAMP production in vitro by isoproterenol was normal in the heart in the early stages of endotoxycosis but was attenuated by 3 hours into the experimental period. Basal levels of cAMP content after a 30 minute incubation in buffer medium were not different at any time during the endotoxycosis. It is concluded that the early decrease in cAMP content in the in situ endotoxic ventricle is not due to alterations in production of cAMP, but rather due to increased degradation, possibly by insulin stimulation of

phosphodiesterase. Late decreases in cAMP content are most likely due to a shortage of available substrate and/or a decrease in the ability to stimulate production via beta-receptor mechanisms.

Since alterations in cAMP production may have been associated with the changes in the enzyme system responsible for generation, the second project focused on myocardial adenylate cyclase activation during endotoxiosis. Stimulation of adenylate cyclase activity by NaF or GppNHp was not different in ventricular membranes from endotoxic rats as compared to controls at any time during the experimental period. These agents stimulate catalytic activity via the guanine nucleotide regulatory protein and bypass the receptor. When the beta-adrenergic receptor agonist, isoproterenol, was used to stimulate adenylate cyclase activity there was no difference between control and endotoxic preparations at 0.5 or 3 hours. At the agonal stage, myocardial adenylate cyclase in the endotoxic membranes was 3 times less sensitive to isoproterenol stimulation than control membranes and had a decreased maximum response. It is concluded from this data that the guanine nucleotide regulatory protein, the catalytic unit of adenylate cyclase, and their interaction are not altered during endotoxiosis, but that receptor-mediated mechanisms are modified.

The third project assessed beta-adrenergic receptor characteristics during the endotoxic episode. Antagonist binding with DHA did not uncover any differences between control or endotoxic

ventricular membrane beta receptors at any time after endotoxin administration. Neither the affinity constant nor density of receptors was affected by endotoxemia. Analysis of agonist binding to receptors, as determined from isoproterenol competition curves, indicated that there was a decreased ability of the agonist to promote the high affinity state of the receptor in the endotoxic membrane in the terminal stages. Formation of the ternary complex is an essential intermediate for coupling of the receptor with adenylate cyclase (23,82). It is concluded that there is no "down regulation" of receptors during endotoxemia, however there is a decrease in receptor coupling to the adenylate cyclase. This uncoupling is responsible for attenuated isoproterenol-stimulation of adenylate cyclase.

These experimental findings are interpreted as evidence that the modifications of receptor mechanisms in the endotoxic rat ventricle are due to agonist induced desensitization. Sympathetic nerve activity to the heart and circulating plasma catecholamines are known to be greatly increased during endotoxemia (63,105). Furthermore, rats infused with catecholamines for equivalent periods develop receptor changes similar to those observed in the present study (18). Such hearts developed a decreased functional response to endogenous catecholamines without decreases in receptor number.

It is difficult to substantiate the theory of desensitization in the endotoxic rat. Infusion of exogenous catecholamines in vivo to stimulate cardiac receptors is problematic. First of all, reflex

mechanisms may be stimulated that alter endogenous catecholamine and acetylcholine release. Increased catecholamine release as a response to vasodilation would cause an overestimation of the sensitivity to the infused drug, since the stimulation from two sources would summate. Withdrawal of sympathetic activity during vasoconstriction would cause an underestimation of sensitivity since tonic release is depressed. Acetylcholine, that is also released when blood pressure increases, can antagonize catecholamine stimulated mechanisms and would thereby cause a decreased sensitivity to the exogenous beta-adrenergic agonist. Secondly, the effects of an infusion of agonist may be masked by plasma levels of catecholamines that are already exaggerated in the endotoxic state. Protection of cardiac beta receptors to prevent overstimulation is also difficult. Sympathectomy and adrenal demedullation (70), ganglionic blockade (unpublished observations) or beta receptor blockade (13) sensitize rats to endotoxin lethality, such that the animals die within minutes of administration. It may be possible to partially block receptors with low doses of antagonists and/or administer the antagonist after the initial hypotension is corrected. However, even apparently minor perturbations may compromise sympathetic support necessary for adequate myocardial performance. The use of isolated hearts or tissues from endotoxin-treated animals is an attractive alternative, however removal from the endotoxic environment may be ambiguous since other factors may be acting on the heart in situ.

Further evidence in support of the desensitization phenomenon is that the myocardial beta receptors in terminal endotoxemia exhibit

characteristics similar to both homologous and heterologous beta receptor desensitization. Homologous desensitization is defined as a specific loss of catecholamine stimulated adenylate cyclase activity and demonstrates both the uncoupling and receptor loss phenomena. Alterations in receptors comprise the major lesions in such cases. In contrast, heterologous desensitization is characterized by a loss in sensitivity to  $F^-$  and GppNHpp as well as a decreased responsiveness to catecholamines, but no loss of receptors (127). These definitions are reflective of data acquired from cultured cell lines and erythrocytes, however the mechanisms of myocardial desensitization have not been as well characterized. Desensitized beta receptors in the present study show a loss of catecholamine stimulated activity and no loss of  $F^-$  or GppNHpp stimulated activity which is similar to homologous desensitization mechanisms, however there is no loss of receptors as with heterologous desensitization. However the time course of the endotoxemia may not have been long enough for receptor down-regulation to develop.

The myocardial beta-receptor changes and the subsequent decrease in responsiveness to catecholamines are not the initiating factors in the myocardial dysfunction of endotoxemia, but such changes may contribute to the pathophysiology of circulatory shock. Therefore, the following schema of events, leading to myocardial failure in shock, is proposed: endotoxin or the septic insult initiates the release of various hormones and mediators. An initial hypotension develops due to the direct effects of endotoxin, a mediator, or histamine or a combination of all three. Sympathetic mechanisms are activated

neurogenically, by endotoxin directly, or by a mediator. The hypotension is compensated and sympathetic activity remains elevated, possibly to excess, to maintain blood pressure and for general support of an organism under stress. The signal that stimulates sympathetic activation after the hypotensive phase is not clear. Myocardial dysfunction is perpetuated directly or indirectly by mediators of endotoxiosis. Factors contributing to the myocardial depression are decreases in contractile protein activation, calcium dyshomeostasis, decrease in energy substrate availability, and electrophysiological changes. As heart function continues to decline sympathetic support is increasingly important and sympathoadrenal activity is increased accordingly. The catecholamines initially serve a protective role, but eventually concentrations of catecholamines at the receptor also reach supranormal levels. Exposure of the receptors to these concentrations for a prolonged period then desensitizes the receptor. Therefore, the failing heart is not supported as well and myocardial performance is no longer sufficient to meet the cardiovascular requirements of an organism that is also metabolically and immunologically stressed. Death follows as a result of cardiovascular, metabolic, and immunologic collapse.

It is important to note that sympathetic activation in the endotoxic rat is grossly exaggerated in comparison to activation in other stress states (26,27,111). The stimulus that is responsible for the hyperactivation is ultimately responsible for the transition of beneficial to detrimental effects of adrenergic stimulation on the heart. By some unknown mechanism the myocardial membrane recognizes that



the adrenergic stimulation is greater than necessary and in order to decrease activation of intracellular processes the receptors are desensitized. Unfortunately, myocardial contractility is decreased during endotoxemia and the loss of receptor activation compromises contractility further. It is probable that myocardial performance could be supported more adequately if plasma catecholamines did not reach such exaggerated levels. In agreement with this concept, rats or patients that had relatively smaller increases in plasma catecholamines appear to be more able to survive an endotoxic or septic insult (4,63).

In the case of the rats it appears that the lower levels of catecholamines correlate with an increased time to death but most animals eventually die (63). This suggests that it may take longer for desensitization and myocardial dysfunction to occur in the endotoxic animals with lower concentrations of plasma catecholamines and/or that mechanisms other than cardiovascular collapse are responsible for the death of the animal. Relevant to human sepsis it would appear that protection of beta-adrenergic receptors with antagonists, while stimulating contractility with non-adrenergic agents and supporting metabolic mechanisms could be beneficial to survival.

Further investigation of beta-adrenergic mechanisms in endotoxemia should aim at determining the role catecholamines or other mediators have in causing the receptor changes observed in the present study and for methods to protect the beta-adrenergic mechanisms. Desensitization of myocardial adenylate cyclase may be non-specific in

that all normal activators show losses similar to catecholamines. Therefore, it would also be important to determine whether other substances that stimulate myocardial adenylate cyclase, such as glucagon or histamine, become less effective and the mechanisms involved.

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## APPENDIX A

### METHODS IN DETAIL

#### A. Animals

Healthy male Holtzman rats (derived from the Sprague-Dawley strain) of 300-400 grams body weight were used in all experiments. Rats were chosen for the studies due to the ease of handling and the extensive data base regarding the patho-physiology of sepsis. Upon receipt from the supplier (Holtzman Company, Madison, Wisconsin), animals were maintained in the Animal Research Facility (Stritch School of Medicine) for at least one week prior to experimentation. This period allowed the animals to recover from the stress of shipping and adjust to the 12 hour light-dark cycle (light from 0700 to 1900 hours) and other environmental conditions. Rats were housed three or four per cage (cage dimensions: 16" length, 9.5" width, and 7" depth). Ambient temperature was approximately 22°C and the humidity was 30%-50%. Animals were maintained on Wayne Lab Blox and tap water ad libitum. Rats had free access to food at all times prior to experiments.

#### B. Model of Sepsis

The endotoxin model of a gram-negative septic insult was

employed in all experiments. Salmonella enteritidis endotoxin, lipopolysaccharide B, prepared by the Boivin method (trichloroacetic acid extraction) was purchased from Difco Laboratories, Detroit, Michigan. The same batch (#703252) of endotoxin was used in all experiments. Endotoxin was prepared daily in 0.9% saline at a concentration of 5 mg/ml. Rats were lightly anesthetized with ether (Mallinkrodt) and received 16.7 mg/kg endotoxin intravenously via the dorsal vein of the penis. This dose of endotoxin produced 80-90% lethality in 24 hours, in agreement with previous studies (). Control animals received a comparable volume of saline. All experiments were initiated between 0700 and 0930 hours to reduce the influence of any diurnal factors such as plasma corticosteroid concentration (). After administration of endotoxin or saline animals were placed in a shoe box cage (4/cage) and followed for a predetermined time or until a shock syndrome ensued. Water was available ad libitum. The terminal stage of shock was considered to be that time when the animal lost its righting reflex.

### C. Ventricular slice preparation

To determine the ability of a beta-adrenergic receptor agonist to stimulate cyclic adenosine-3',5'-monophosphate (cAMP) production in the endotoxic rat heart, in the absence of other mediators, a ventricular slice preparation was employed.



Rats were decapitated at 0.5 and 3.0 hours after endotoxin or saline administration or when they lost their righting reflex. Saline treated animals were sacrificed at a time comparable to those animals that were in shock. Times were chosen based on initial experiments that observed the time course of endotoxemia. Hearts were rapidly removed and placed in 4°C Krebs-Ringer-Bicarbonate buffer (KRB) which contained NaCl, 118.5 mM; KCl, 4.8 mM;  $\text{KH}_2\text{PO}_4$ , 1.2 mM;  $\text{MgSO}_4$ , 1.2 mM;  $\text{CaCl}_2$ , 3.6 mM; glucose, 5.5 mM; and  $\text{NaHCO}_3$ , 23.8 mM. The KRB was previously gassed for 30 min with 95% $\text{O}_2$ -5% $\text{CO}_2$  and the pH adjusted to 7.4 with HCl or NaOH. The atria, large vessels and adipose tissue were trimmed and the ventricles sliced in half transversely such that each half contained both right and left ventricle. The ventricles were sliced in a cold room with a Stadie-Riggs microtome (A. Thomas and Co.). Slices were approximately 0.5 mm thick. The slices were randomly placed in six 10 ml beakers that contained 2.4 ml of oxygenated KRB, 37°C, pH 7.4. Each beaker contained 3-4 slices of ventricle with a total weight of 75-100 mg. The slices were incubated for 15 min in a Dubnoff metabolic shaker while being continuously gassed with 95% $\text{O}_2$ -5% $\text{CO}_2$ , to recover from the previous manipulations and reach an basal state. After the incubation period, 100  $\mu\text{l}$  of  $2.5 \times 10^{-5}\text{M}$  isoproterenol (final concentration of  $10^{-6}\text{M}$ ), prepared in 0.1% ascorbic acid to prevent oxidation of the catecholamine, was added to 2 beakers. Two other beakers received 100  $\mu\text{l}$  of 0.1% ascorbic acid and the last two received 100  $\mu\text{l}$  of KRB. Forty-five seconds after the additions were made the slices were removed from the beakers, blotted dry, and frozen with brass clamps previously cooled in liquid nitrogen. The samples were stored at -195°C, in liquid

nitrogen, until assayed for cAMP.

Preliminary experiments tested various doses of isoproterenol and times of exposure to the drug. The dose used ( $10^{-6}$ M) was found to stimulate cAMP production maximally and the time (45 sec) was sufficient to develop this response.

#### D. Implantation of Cannulae

In order to collect blood samples throughout the time course of endotoxemia a group of rats were instrumented as follows. Rats were anesthetized with 4% halothane in oxygen for induction and maintained on 1.75% halothane in oxygen. A 2 cm long incision was made just lateral to the ventral midline on the left side of the neck. The sternohyoid and sternomastoid muscles were reflected and the left common carotid artery exposed. A cannula fashioned from PE-50 polyethylene tubing (Clay-Adams) was inserted into the carotid artery until the tip entered the descending aorta (this was verified at autopsy). The cannula was exteriorized in the mid-scapular region and the incision was closed with surgical staples. These animals were allowed to recover approximately 30 minutes before the experimental procedure was initiated. At that time they were treated exactly as the non-cannulated animals (see Section B), except that they were placed one per cage in order to eliminate tangling and chewing of cannulae. Cannulae were filled with heparinized saline (100 U/ml).

Blood samples of 0.8 ml were taken from the carotid cannula at predetermined times and replaced by a similar volume of saline. Samples were placed in 1.5 ml microcentrifuge tubes containing 16  $\mu$ l of a solution containing 90 mg/ml ethyleneglycol-bis-(B-amino-ethyl ether) N,N'-tetraacetic acid (EGTA) and 60 mg/ml glutathione (pH 6-7). The tubes were held on ice until centrifuged at approximately 10,000 g in a Eppendorf Model 5414 microfuge. The plasma was stored at  $-40^{\circ}\text{C}$ , in a Revco freezer, until assayed for catecholamines.

#### E. Plasma Catecholamine Determination

Concentrations of circulating plasma epinephrine and norepinephrine were assayed according to the method of Passon and Peuler (1) as modified by Peuler and Johnson (2) by use of a kit supplied by The Upjohn Company, Kalamazoo, Michigan. This is a single isotope radioenzymatic method that simultaneously measures epinephrine and norepinephrine in plasma samples of 50  $\mu$ l or less with a sensitivity of 1 pg (20 pg/ml plasma). This technique is based on the use of the isolated enzyme catecholamine-O-methyltransferase (COMT) to transfer a radioactive methyl group from S-adenosyl-L-methionine (SAM) to an endogenous catecholamine acceptor molecule to form a O-methyl catecholamine derivative. Norepinephrine is converted to  $^3\text{H}$ -normetanephrine in the presence of SAM( $^3\text{H}$ -methyl) and epinephrine is converted to  $^3\text{H}$ -metanephrine.

Fifty  $\mu$ l aliquots of plasma were added to incubates containing

100 mM Tris, 30 mM  $\text{MgCl}_2$ , 10 mM EGTA, 10  $\mu\text{l}$  of COMT solution, 1 mM reduced glutathione, 0.1 mM benzylhydroxyamine (to inhibit dopa decarboxylase), 5  $\mu\text{Ci}$  SAM( $^3\text{H}$ -methyl, 4.3-5.5 mM). Total volume was 100  $\mu\text{l}$  and pH was approximately 8.1-8.3. One hundred picograms of norepinephrine and epinephrine were added to duplicate incubation mixtures, also containing 50  $\mu\text{l}$  of sample plasma, as internal standards. Reagent blanks contained the incubation mixture, however 50  $\mu\text{l}$  of vehicle, containing EGTA and glutathione, were substituted for the plasma.

The samples were incubated in a shaking water bath at  $37^\circ\text{C}$  for 60 minutes. The reaction was stopped by the addition of 50  $\mu\text{l}$  of a solution containing 800 mM boric acid, 80 mM ethylenediamine tetraacetic acid-disodium salt ( $\text{EDTA-Na}_2$ ), and 4 mM each of metanephrine and normetanephrine in 1N NaOH. The resulting solution (pH 10.0) was mixed vigorously for 15 s with 2 ml of toluene-*n*-amyl alcohol (3:2, v:v). The  $^3\text{H}$ -methoxycatecholamine derivatives are much more soluble in the organic mixture than in the aqueous reaction mixture. The tubes were centrifuged at 800 g for two minutes in an International Equipment Company (IEC) Model UV centrifuge to separate the organic and aqueous phases. The aqueous phase was rapidly frozen in an ethanol/dry ice bath and the organic phase decanted into a tube containing 100  $\mu\text{l}$  of 0.1 M acetic acid. The  $^3\text{H}$ -methylcatecholamine derivatives were partitioned into the aqueous phase by mixing for 15 s. The decrease in pH effected by the addition of acetic acid greatly reduced the solubility of the derivatives in the organic phase. The tubes were centrifuged at 800 g

for 2 min, the aqueous phase was quick-frozen and the organic phase was discarded. The acetic acid phase was thawed and to it was added 1 ml of the toluene-isoamyl alcohol mixture. After centrifugation and freezing the organic phase was again discarded. One hundred  $\mu$ l of ethanol was then added to the thawed acetic acid extract and the solution was applied to a silica gel thin layer (Uniplate, Analtech) by use of 250  $\mu$ l Hamilton syringes and a thin layer chromatography (TLC) Multispotter (Analytical Instrumentation Specialties) capable of applying 14 samples simultaneously. The plates were developed for 30-40 min in a solution of t-amyl alcohol/toluene/40% methylamine solution, 12:4:5. The plates were dried thoroughly using a hot air blow-dryer and two zones were visualized under 254 nm UV light. The upper zone contained metanephrine and the lower zone contained normetanephrine. Each zone was then scraped from the plate into a correspondingly numbered scintillation vial. The assay was generally interrupted at this point and the vials stored at 4°C overnight. The procedure was completed the following morning.

The assays for norepinephrine and epinephrine were identical. One ml of 0.05 M ammonium hydroxide was added to each vial to elute the derivatives from the silica gel. The  $^3\text{H}$ -catecholamine derivative was then oxidized to  $^3\text{H}$ -vanillin by the addition of 50  $\mu$ l of sodium periodate followed 5 min later by the addition of 50  $\mu$ l glycerol (10%, v:v). The solution was acidified by the addition of 1 ml of 0.1 M acetic acid and vigorous mixing. Ten ml of toluene/Liquiflor (1000:50) was added to each vial and the  $^3\text{H}$ -vanillin was partitioned into the organic phase with 30 s of shaking. Liquiflor (New England Nuclear) is a

PPO-POPOP toluene concentrate.

All scintillation vials were dark adapted and then counted for radioactivity for 10 min in an Isocap/300 scintillation counter (Searle-Analytical) without quench correction.

#### Calculation of Sample Catecholamine Content

The calculations for each catecholamine are exactly the same and proceed as follows:

1. The counts per minute (CPM) for each set of duplicates is averaged.
2. The CPM that are due to the addition of standard are determined and averaged so that one value is used for all subsequent calculations.
3. The CPM for the blank is subtracted from the CPM of each sample and this value is divided by the CPM due to the addition of standard. Thus a ratio of CPM of unknown catecholamine concentration to CPM of known catecholamine concentration is calculated.
4. The ratio of unknown to known is multiplied by the concentration of standard in the assay and thus the sample concentration is determined. The final value is expressed as picograms of catecholamine/ml of plasma.

#### F. Determination of Tissue Catecholamine Concentrations

## Tissue Acquisition

Rats were decapitated and the thorax and abdomen opened. The heart was excised and the ventricles separated from the atria and large vessels. The ventricles were blotted free of blood and frozen between brass clamps that were pre-cooled in liquid nitrogen. The tissue was then immersed in liquid nitrogen to facilitate the freezing process. The spleen was similarly excised and frozen. The adrenal glands were frozen as a pair. All tissues were stored at  $-195^{\circ}\text{C}$  until assayed for catecholamines.

## Catecholamine Extraction

The extraction and purification procedure is based on the methods of Shaw (3) as modified by Lund (4). Tissues were weighed frozen on a Roller-Smith balance and placed in 10 ml of ice-cold 10% trichloroacetic acid (TCA). Each thawed sample was homogenized for 20 s at a setting of 6 with a Brinkmann polytron. The homogenates were centrifuged for 20 min. in an IEC centrifuge Model UV at full speed. The supernatant was decanted into a clean tube and the precipitate discarded. The supernatant was stored at  $4^{\circ}\text{C}$  until purification.

Five ml of the sample were pipetted into a 50 ml Erlenmeyer flask to which had previously been added 0.5 ml of cold 0.2 M EDTA, 10 ml of cold 0.2 M sodium acetate (NaOAc) and 1 drop of 1% phenolphthalein. The sample was titrated with cold 0.5 N NaOH until it turned slightly pink (pH 8.4). The titrated sample was added to a column which contained a slurry of 0.5 g acid-washed alumina. The custom-made

glass columns were 15 cm long with an inside diameter of 0.5 cm and a 50 ml reservoir. After the sample ran through the column (the eluate was discarded) the alumina was washed with 10 ml of cold distilled water and the water discarded. Excess water was removed from the column tip and 5 ml of 0.2 M acetic acid was added to the column. The resulting eluate was collected for analysis and was stored for up to two weeks at 4°C. In theory, the negatively charged catecholamines at pH 8.4 adsorb to the positively charged alumina and after washing can be eluted with acid. Recovery of catecholamines is routinely 80% using this method ( ).

#### Catecholamine Analysis

The analysis of catecholamines is the basic method of Crout (5) as adapted to the autoanalyzer by Jellinek (6). The series of reactions are as follows. The samples are mixed with 1 M sodium acetate buffer (pH 6.5 or 3.5) to make slightly acidic. The 2-iodo derivative of the catecholamine is formed when oxidized with 0.1 N iodine. The introduction of alkali, in the form of 1 N NaOH, causes the aminochromes to undergo spontaneous tautomerization to highly fluorescent derivatives of 3,5,6-trihydroxyindole. Ascorbic acid (7.5 mM) is added as a reducing agent to remove dissolved oxygen and stabilize these compounds which are unstable in alkaline solution.

The relative fluorescence of these compounds was then measured with a Turner fluorometer which was equipped with a flow cell, a primary filter of the narrow pass variety at 436 nm and a secondary sharp cutoff filter above 510 nm. The fluorometer was also interfaced with a Beckman



strip chart recorder.

The differential quantitation of epinephrine and norepinephrine in the same sample is possible because the degree of oxidation to the corresponding aminochrome by these substances varies with the pH. At a pH of 6.5 both norepinephrine and epinephrine are oxidized, however at a pH of 3.5 only the epinephrine is significantly oxidized.

In addition to analysis at pH 6.5 and 3.5 each sample was also analyzed at a pH of 6.5 with 50 ng/ml norepinephrine added to the buffer and at pH 6.5 with the ascorbate step delayed. The first modification allows for the estimation of fluorescent quench. The second modification produces a blank since the 3,5,6-trihydroxyindole is destroyed before the fluorescence can be determined.

#### Calculation of Tissue Catecholamine Content

The concentrations of norepinephrine and epinephrine in each sample eluate are estimated differentially by solving the following simultaneous equations:

$$(a/b) \times NE + (c/d) \times E = \text{peak height of sample at pH 6.5}$$

$$(e/b) \times NE + (f/d) \times E = \text{peak height of sample at pH 3.5}$$

where:

a = peak height of norepinephrine standard at pH 6.5;

b = concentration of norepinephrine standard;

c = peak height of epinephrine standard at pH 6.5;

d = concentration of epinephrine standard;

e = peak height of norepinephrine standard at pH 3.5;

f = peak height of epinephrine standard at pH 3.5

NE = concentration of norepinephrine in sample;

E = concentration of epinephrine in sample;

Solving the equations gives a value of  $\mu\text{g}$  of catecholamine/ml of eluate. To determine tissue concentration this value is multiplied by the total eluate volume (10 ml + tissue wt. in ml) and divided by the weight of the sample. The final value is expressed as  $\mu\text{g}$  of catecholamine/g of tissue wet wt.

In practice, all data reduction and calculations were performed using a computer program written by Benet J. Pardini, Ph.D, which was converted to Applesoft basic by the author.

#### G. Cyclic AMP determination

All procedures are based on the radioimmunoassay as developed by Steiner (7). The reagents and radioisotope were supplied in kit form by New England Nuclear (Boston, MA).

The basic principle of the radioimmunoassay (RIA) is the competition between radioactive and non-radioactive antigen for a fixed number of antibody binding sites. As increasing amounts of non-radioactive antigen (e.g. standards or unknowns) and a fixed amount of radioactive antigen are allowed to react with a constant amount of

antibody, a decreasing amount of the labelled antigen is bound to the antibody. This relationship can be expressed as a standard curve and the amount of unlabeled antigen in a sample is measured by interpolation from this curve.

#### Preparation of Tissues

Frozen ventricular slices were placed in 2 ml of ice-cold TCA and whole ventricles were placed in 10 ml. All tissues were weighed on Roller-Smith balance prior to homogenization. Tissues were homogenized at 4°C for 20 s with a Brinkmann polytron at a setting of 6. The homogenates were centrifuged in a IEC Model UV centrifuge at 1800 g. The supernatants were decanted and stored at -40°C until purification.

The TCA supernatants were thawed and 1 ml was placed in a 10 ml conical centrifuge tube. The supernatants were extracted four times with 4 ml of ethyl ether saturated with water. The extracted aqueous phase was then frozen and lyophilized. The residue was dissolved in 1 ml of 50 mM sodium acetate buffer, pH 6.2, and used directly in the immunoassay.

#### Cyclic AMP Assay

Reagents were prepared daily to the appropriate specifications. The cAMP standard was reconstituted such that the resulting solution contained 5000 picomoles/ml in 50 mM sodium acetate buffer, pH 6.2. A standard curve was prepared from this solution such that the final amounts of cAMP in the assay tubes were 25.0, 10.0, 5.0, 2.5, 1.0, 0.5, 0.25, 0.1 and 0 picomoles. The lyophilized antiserum complex which

contained rabbit cAMP antibody pre-reacted with sheep anti-rabbit globulin, was reconstituted in distilled water resulting in a solution of 0.1 M sodium phosphate buffer (pH 6.2) containing sufficient antibody to bind 40-50% of the labeled antigen in the absence of unlabeled antigen. The labeled cAMP is a tyrosine methyl ester derivative of 2'-O-succinyl-cAMP (ScAMP-TME), iodinated on the tyrosine moiety. Steiner et al (7) reported that cyclic nucleotides substituted at the 2'-O-position have higher affinity for antibody than unsubstituted cyclic nucleotide.

A 100 ul aliquot of standard or sample was added to 100 ul of ScAMP-TME-( $^{125}\text{I}$ ),  $7 \times 10^{-3}$  uCi (sp. act. 3000 uCi/ug), and 100 ul of antiserum in a 12x75 mm glass culture tube, at room temperature. All samples were assayed in duplicate. The mixture was vortexed, covered with aluminum foil, and allowed to incubate at 4°C for 16-18 hours. In addition, one set of tubes contained only 100 ul of labeled cAMP to determine total counts. This tube was counted without further processing. Another set of tubes contained labeled cAMP and 200 ul of buffer to serve as a blank. This was treated the same as the sample tubes. At the end of the incubation period each tube, except total counts, received 1 ml of ice-cold 50 mM sodium acetate buffer, was vortexed, and centrifuged in the cold for 15 min at 2000 g in a Sorvall RC-5 centrifuge. The tubes were decanted and blotted on absorbent paper. The tubes, containing the precipitated antibody complex, were counted for 1 min in a Searle Analytic gamma counter with a counting efficiency of 80%.

### Calculation of Sample Cyclic AMP Content

The amount of cAMP present in each 100 ul of sample was calculated using a program written in Applesoft basic and performed on an Apple II+ computer. The program employed the following method:

1. The CPM for each set of duplicates was averaged.
2. The average net CPM for all samples was calculated by subtracting the average counts of the blanks.
3. The average net CPM of each standard or sample was expressed as a percent of the average net CPM of the "0" standard, which represents the counts bound to antibody in the absence of added cAMP. This value is the normalized percent bound, B, where  $B = \text{CPM standard} / \text{CPM "0" standard}$ , multiplied by 100 and  $B_0 = 100\%$ .
4. The logit of the percent bound for each standard is calculated ( $\text{logit } B = \ln(B/(B_0 - B))$ ) and a regression of the logit on the log of the standard concentration is fit. This transformation fits the standard curve to a straight line and facilitates the evaluation of each unknown.
5. The sample regression equation is calculated and the logit of percent bound for each sample is fit into the equation to determine the corresponding amount of cAMP present in the sample.
6. The values obtained are then multiplied by the appropriate dilution factor and expressed as pmol cAMP/g tissue wet wt. The dilution factor for whole ventricles was 100, since one

ml out of 10 ml of the homogenate supernatant was lyophilized and 100 ul of 1 ml of the reconstituted sample was used in the assay. The dilution factor for ventricular slices was 20. One ml of two ml of the homogenate supernatant was lyophilized and then 100 ul of 1 ml of the reconstituted sample was used in the assay. In both cases the tissue water was considered negligible compared to the volume of acid in which the tissue was homogenized.

7. The values obtained for the adenylate cyclase assays were multiplied by 8.5 since 100 ul of the final volume of 850 ul was used in the assay. The data was then expressed as pmol cyclic AMP/mg protein-min.

#### H. Determination of Adenylate Cyclase Activity

##### Membrane Preparation

Rats were killed by decapitation with a guillotine (Stoetler Apparatus Company); the hearts were excised, placed in 0.9% saline at 4°C, perfused retrogradely through the aorta with cold saline to remove blood in the coronary vasculature and cleaned of adipose tissue and clots. The ventricles were separated from the atria and frozen between brass clamps that were previously cooled in liquid nitrogen. The tissue was then immersed in liquid nitrogen to complete the freezing process. Tissue was stored at in liquid nitrogen until processed.

Frozen tissue (approximately 150 mg) was weighed on a

Roller-Smith balance and placed in 2 ml of ice-cold grinding buffer (5 mM Tris-HCl, 1mM  $MgCl_2$ , 250 mM sucrose, pH 7.4). Preparations were exposed to two 15 s bursts of a Tekmar Tissumizer at setting 75. The homogenate was passed through four layers of surgical gauze and centrifuged at 48,000 g for 10 min in a Sorvall RC-5 refrigerated centrifuge. The pellet was resuspended in 20 volumes (v/original wet wt.) of ice-cold incubation buffer (75 mM Tris-HCl, 15 mM  $MgCl_2$ , pH 7.4) with the Tissumizer at low speed. The pellets are was recentrifuged and resuspended twice. The final suspension contained approximately 2 mg of membrane protein per ml.

#### Adenylate Cyclase Assay

This procedure was based on the methods of O'Connor et al (8). Approximately 100 ug of membrane were added to a total volume of 150 ul of 75 mM Tris-HCl (pH 7.4), 8 mM KCl, 7.5 mM theophylline, 0.4 mM dithiothreitol, 0.4 mg/ml bovine serum albumin, 0.7 mM EGTA, 6 mM  $MgCl_2$ , 1mM ATP, 2.5 mM creatine phosphate, 0.075 mg/ml creatine phosphokinase, and 1.0 mM ascorbic acid in a 1.5 ml polyethylene microcentrifuge tube (Sarstedt) on ice. The tubes were incubated for 20 min at 30°C in a Dubnoff metabolic shaker. In certain assay conditions either 8 mM NaF,  $10^{-4}$  M Gpp(NH)p (guanylyl-imidodiphosphate),  $10^{-4}$  M GTP (guanosine triphosphate), or  $10^{-4}$  M GTP plus  $10^{-9}$  to  $10^{-3}$  M isoproterenol were included. After the incubation period the tubes were returned to the ice bath and the reaction was terminated by the addition of 700 ul of cold 1.0 mM EDTA, 50 mM sodium acetate (pH 6.2). The assay tubes were centrifuged for 10 min at 10,000 g in a Eppendorf Microfuge Model 5414

and the supernatant was decanted into fresh tubes and stored at  $-40^{\circ}\text{C}$  until assayed for cAMP. One hundred  $\mu\text{l}$  of supernatant were used directly in the assay as described above (see Section G).

## I. Beta-Adrenergic Receptor Characterization

### Membrane Preparation

Suitable cardiac membrane preparations were prepared by a modification of the method of Baker et al (9). Ventricular tissue was acquired as explained in section H.

Frozen ventricles (approximately 750 mg) were weighed on a Roller-Smith balance, minced with a sectioning blade, and placed in 10 ml of ice-cold grinding buffer (5 mM Tris-HCl, 1 mM  $\text{MgCl}_2$ , 250 mM sucrose, pH 7.4). The tissue was allowed to thaw then homogenized by means of two 15 s bursts at setting 6 on a Polytron homogenizer ST-10 (Brinkmann Instruments). The homogenizer was rinsed with 5 ml of grinding buffer and this was added to the homogenate. An equal volume of cold 1 M KCl (15 ml) was added to the homogenate and the mixture was continuously stirred on ice for 15 min. This procedure extracts contractile proteins and decreases non-specific binding of ligand. The homogenate was filtered through four layers of surgical gauze and centrifuged at 48,000 g for 15 min. The supernatant was discarded and the pellet resuspended in 25 volumes (v/w) of incubation buffer (75 mM Tris-HCl, 15 mM  $\text{MgCl}_2$ , 1 mM ascorbic acid, pH 7.4) and recentrifuged and resuspended twice. The final suspension had a final protein



concentration of 0.6-1.0 mg/ml incubation buffer.

### Binding Assays

This procedure was based on the methods of Lurie et al (10). The membrane preparation (250 ul) was added to 12x75 mm glass tubes on ice, containing ( $^3\text{H}$ )-dihydroalprenolol (DHA, sp. act. 35.6 Ci/mmol, NEN) or DHA and (-)isoproterenol ( $10^{-3}$  M) in a final volume of 500 ul of incubation buffer. All assays were performed in duplicate. The tubes were vortexed briefly and incubated at 25°C for 30 min in a shaking water bath. When the incubation period was complete, binding was terminated by adding 4 ml of ice-cold buffer (75 mM Tris-HCl, 15 mM  $\text{MgCl}_2$ , pH 7.4) to each tube and the samples were filtered (24 at a time) over GF/C glass-fiber filters (Whatman) using a cell harvester (Brandel Model M24). Each filter was washed 3 times with 3 ml of ice-cold buffer. The filters were placed in 5 ml mini-scintillation vials (Research Products International) and allowed to dry. Three and one half ml of scintillation fluid (3a70b, RPI) were added to each vial and the radioactivity was counted in a Packard Tri-Carb 460 liquid scintillation counter. Disintegrations per minute for each sample were calculated by the standard channels ratio method. This calculation was performed by a program operated by the counter.

Specific binding (DHA bound to B-adrenergic receptors) was determined by subtracting DHA binding in the presence of  $10^{-3}$  M (-)isoproterenol from DHA binding in the absence of isoproterenol. This concentration of isoproterenol was found to maximally inhibit DHA

binding in DHA-isoproterenol competition curves. Specific binding was generally 65-70% of total binding at the  $K_d$  for DHA. Specific binding was shown to be saturable and linearly related to protein concentration. Binding equilibrium was observed to occur within 10 min from initiation of the reaction. Total ligand bound was normally 1-3% Of the free ligand.

#### Saturation Isotherms

Heart membranes were incubated as indicated above in the presence of increasing concentrations of DHA (0.25-10 nM, approximately 10,000-450,000 DPM) in the presence or absence of 1 mM isoproterenol. Specific binding was measured as indicated above after a 30 min incubation period. The results were analyzed according to the method of Scatchard (11). Beta-adrenergic receptor density and antagonist affinity were derived from this data.

#### Competition Curves

Heart membranes were incubated for 30 min as indicated above in the presence of a fixed concentration of DHA (2 nM) and increasing concentrations of (-)-isoproterenol ( $10^{-9}$ - $10^{-3}$  M). Isoproterenol was used since it is considered not to have significant alpha-adrenergic receptor binding capacity. Under these conditions maximal binding represented 1-3% of the total radioactivity added. Competition curves were analyzed by computer curve fitting techniques (13,14). A one or two class of binding sites model was accepted on the basis of the best fit as determined by the curve fit. Binding characteristics of the adrenergic

agonist, as opposed to antagonist, were derived via this analysis.

#### Calculation of Beta-adrenergic Receptor Density and Antagonist Affinity

The following abbreviations will be used in this discussion:

L = concentration of free radioligand

R = concentration of unoccupied binding sites

RL = concentration of receptor bound with radioligand

$R_T$  = total receptor concentration

$K_d$  = equilibrium dissociation constant

In a saturation experiment the receptor concentration is held constant and RL is determined at equilibrium as a function of L. By the Law of Mass Action

$$R + L = RL \quad \text{and}$$

$$K_d = (R * L) / RL \quad *=\text{multiplication}$$

Since the the total receptor concentration must be a sum of the free and bound concentrations

$$R_T = R + RL$$

combining the last two equations and solving for RL gives

$$RL = (R_T * L) / (K_d + L)$$

This equation describes a rectangular hyperbola and displays the saturability of the binding. In order to analyze this data it is much easier to represent it mathematically as a linear relationship, thus the Scatchard (11) or Rosenthal (12) transform is calculated. The parameters of this equation are usually represented by a different set of symbols: RL = B (Bound); L = F (Free); and  $R_T = B_{\max}$  (maximal number of binding sites). By rearranging the last equation and inserting the new symbols

the equation becomes

$$B/F = (-B/K_d) + (B_{\max}/K_d)$$

A plot of  $B/F$  on the ordinate versus  $B$  on the abscissa produces a straight line (when binding occurs at a single site) with a slope of  $-1/K_d$  and a X-intercept of  $B_{\max}$ . Data that does not model to a one site fit is analyzed by the non-linear curve fitting program described below.

In practice the data is prepared for this transformation in the following manner:

1. Averages are calculated for each set of duplicates.
2. Specific bound DPM are calculated by subtracting non-specific bound DPM (DPM in the presence of isoproterenol) from total bound (no isoproterenol) for each concentration of DHA.
3. Specific bound DPM are converted to femtomoles of DHA/mg of membrane protein by multiplying by a conversion factor (fmol/DPM) and dividing by the membrane protein in each tube. Thus "B" is calculated.
4. Subtracting specific bound DPM from the total DPM added to the tube gives free DPM. Free DPM is converted to concentration of free DHA by multiplying by the conversion factor and dividing by the volume of the assay (0.5 ml). Thereby "F" is calculated.
5.  $B/F$  is calculated for each concentration of DHA.
6. A regression of  $B/f$  on  $B$  is fit. The negative reciprocal of the slope of the line is  $K_d$  and the X-intercept is  $B_{\max}$ .

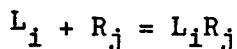
Scatchard analysis is performed by using a Visi-Calc program on

the Apple II+ to obtain B and B/F values and Hewlett-Packard HP-11C calculator for fitting the regression line.

### Competition Curve Analysis

This analysis was performed for the most part with the LIGAND program of Munson and Rodbard (13), as converted to Applesoft basic by Martin H. Teicher, Ph.D, on an Apple II+ computer. The program listing was acquired from the Biomedical Computing Technology Information Center, Vanderbilt Medical Center.

LIGAND is a non-linear model fitting program which will analyze the results of ligand-binding studies using an exact physical chemical model with appropriate statistical methodology. No computational approximations or simplifications are used. Rather than transforming the data, the program calculates bound ligand concentration as a function of the total ligand concentrations for each ligand in the system. The mathematical model describes the reactions of  $n$  ligands,  $L_i$ , binding to  $m$  classes of sites,  $R_j$ .



for

$i=1$  to  $n$ ,  $j=1$  to  $m$ ,

each with an affinity constant:

$$K_{ij} = B_{ij}/F_i * E_j \quad (A1)$$

where  $F_i$  represents the free concentration of ligand  $i$ ,  $E_j$  is the concentration of empty receptor  $j$ , and  $B_{ij}$  is the concentration ligand  $i$  bound to receptor  $j$ . Empty receptor,  $E_j$ , is defined by the conservation

of mass equation for receptors:

$$R_j = E_j + B_{ij}$$

for

$$j=1 \text{ to } m \quad (A2)$$

where  $R_j$  is the total concentration of receptor  $j$ . Free ligand concentration,  $F_i$ , is defined by the equation for conservation of ligand:

$$L_i = F_i + B_{ij}$$

for

$$i=1 \text{ to } n \quad (A3)$$

Here,  $L_i$  is the total concentration of ligand  $i$ . Solving Eq. (A1) for  $B_{ij}$  and substituting for  $B_{ij}$  into Eqs. (A2) and (A3) yields

$$R_j = E_j + K_{ij}E_jF_i$$

for

$$j=1 \text{ to } m \quad (A4)$$

$$L_i = F_i + K_{ij}E_jF_i$$

for

$$i=1 \text{ to } n \quad (A5)$$

Solving Eq. (A4) for  $E_j$  yields

$$E_j = R_j / (1 + K_{ij}F_i)$$

for

$$j=1 \text{ to } m \quad (A6)$$

which may then be substituted into Eq. (A5) to give

$$L_i = F_i + ((K_{ij}R_jF_i)/(1 + K_{ij}F_i))$$

for

$$i=1 \text{ to } n \quad (A7)$$

Equation (A7) is then solved numerically for  $F_i$ , given  $L_i$ ,  $R_j$ , and the matrix of values  $K_{ij}$ . In turn, the solution for  $F_i$  may be substituted into Eq. (A6) to find  $E_j$ , and then, using Eq. (A1), calculate  $B_{ij}$ . Thus the bound concentration is calculated as a function of the total ligand concentrations for the ligand,  $B_{ij}(L_1, L_2, \dots, L_n)$  given the values of the binding parameters  $K_{ij}$  and  $R_j$ .

Since this program calculates binding for any number of ligands at any number of binding sites it is especially suitable for determining the characteristics of agonist binding using competition assays. In practice, isoproterenol binding characteristics are determined as a function of the ability to compete with DHA binding. By knowing the binding characteristics of DHA without isoproterenol (see above) and the binding of DHA at every level of isoproterenol the program can then determine the binding characteristics of isoproterenol. Additionally the data can be modelled to different fits. That is, it can be determined whether isoproterenol is binding to one or more sites, the density of each site, and their affinities. As additional parameters (binding sites, etc.) are added to the model, the goodness of fit will tend to improve. The program will test whether the increase of goodness of fit for a model with additional parameters is significantly more than is expected on the basis of chance alone. The test is based on the "extra sum of squares" principle. An F ratio is calculated from the residual sums of squares (SS) of the deviations to the fitted curves and the degrees of freedom (df):

$$F = ((SS_1 - SS_2) / (df_1 - df_2)) / (SS_2 / df_2)$$

If the F value exceeds the tabulated value for the appropriate degrees of freedom and probability level the second fit is considered a significant improvement over the first.

Competition curves were also analyzed by the ALLFIT program of DeLean, Munson and Rodbard (14) in the Applesoft version converted by Martin H. Teicher. This program analyzes groups of sigmoidal dose response curves. The model used is the four parameter logistic equation:

$$Y = ((a-d)/(1 + (X/c)^b)) + d$$

where X and Y are the dose and the response respectively and a,b,c,d are the four fitted parameters: response at zero dose (a), slope factor (b), 50% maximally efficient dose or EC<sub>50</sub> (c) and response at "infinite" dose (d). This program was used independently to determine the aforementioned parameters for each competition curve, however comparison of curves sharing common parameters is also possible.

#### J. Protein Determination

This assay is based on the methods of Bradford (15) and is performed using the appropriate solution as supplied by Bio-Rad Laboratories. The assay is based on the observation that Coomassie Brilliant Blue G-250 exists in two color forms, red and blue. The red form is converted to the blue form upon binding of the dye to protein. The protein-dye complex was found to have a high extinction coefficient which affords great sensitivity in measurement of the protein.



One hundred microliters of standard (20-100 ug of bovine serum albumin) or sample were placed in 16x100 mm glass test tubes at room temperature. One hundred microliters of sample buffer (75 mM Tris-HCl, 15 mM MgCl<sub>2</sub>, pH 7.4) were added to a separate test tube to serve as the blank (0 absorbance). To each tube was added 5.0 ml of Bradford dye reagent containing Coomassie Brilliant Blue G-250 0.01% (w/v), 4.7% (w/v) ethanol, and 8.5% (w/v) phosphoric acid. The mixtures were vortexed gently and binding allowed to reach equilibrium (5 min). The absorbance of each sample and standard was measured with a Beckman DB-G grating spectrophotometer at 595 nm. The absorbance for the standards and samples were corrected for the blank. A standard curve was prepared by plotting absorbance versus concentration of the standards. Protein concentrations of the samples were determined from the standard curve. The standard curve was found to be linear between 20 and 100 ug and all samples were appropriately diluted to fall within this range.

#### K. Plasma Glucose Analysis

Blood was collected from the neck wound after decapitation in 1.5 ml heparinized microcentrifuge tubes. The samples were centrifuged for 3 min in an Eppendorf Model 5414 microfuge at 10,000 g. The plasma was transferred with Pasteur pipettes to clean 400 ul microfuge tubes and stored at -20°C until assayed.

Plasma glucose concentrations were determined with a Yellow Springs Instruments Model 23A Glucose analyzer. The analyzer is

basically an oxidase enzyme hydrogen peroxide sensor as developed by Dr. Leland C. Clark and is capable of measuring glucose in plasma, serum, whole blood, and other biological and non-biological fluids.

Glucose in the vicinity of the probe diffuses through the outer layer of the membrane, which is a polycarbonate material with pores large enough to pass glucose, oxygen, hydrogen peroxide, water and salt but small enough to exclude cells and enzymes. The glucose comes in contact with glucose oxidase which is immobilized in a resin between the inner and outer layer. The glucose is converted to gluconic acid and hydrogen peroxide by this enzyme. The hydrogen peroxide diffuses through the inner cellulose acetate layer, coming in contact with the platinum anode. The hydrogen peroxide is oxidized to liberate hydrogen ions, oxygen, and electrons. The oxygen is then reduced to water by a silver cathode. These two reactions yield a current proportional to the quantity of the hydrogen peroxide diffused, which is directly proportional to the amount of glucose in the sample. The concentration of glucose in the sample is determined from the amount of glucose measured and the volume of the sample, which is constant for all samples. The glucose analyzer was calibrated daily against known standards. Samples were analyzed in duplicate and the average of these is reported as mg/100 ml plasma.

#### L. Statistical Analysis

Statistical significance was determined by several methods. An independent student's t test was used to compare two groups. The

comparison of more than two groups was performed with an independent analysis of variance. The least significant difference technique was used for mean separation after significant F tests were completed. Analysis of covariance and linear regression were used to compare dose response curves. A p value of less than 0.05 was considered as the minimum level to achieve significance.

All analysis was performed with programs modified for the Apple II+ computer by either the author or David V. Defily.

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APPROVAL SHEET

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The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

April 16, 1985  
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